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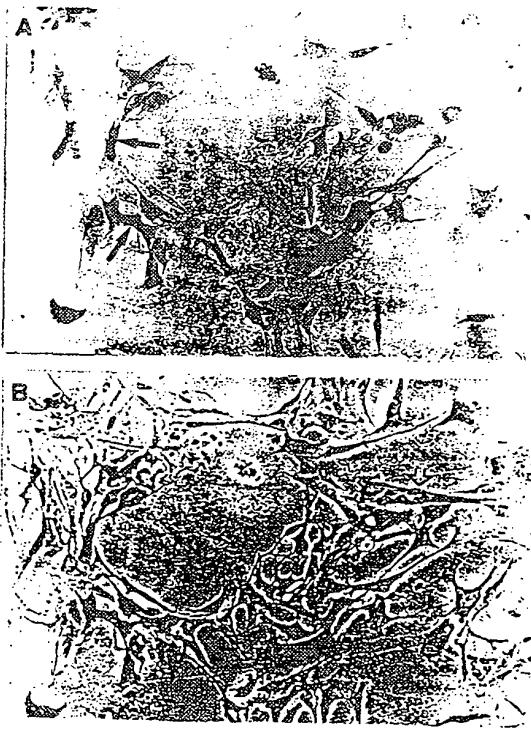
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(54) Title: MAMMALIAN MULTIPOTENT NEURAL STEM CELLS

(57) Abstract

The invention includes mammalian multipotent neural stem cells and their progeny and methods for the isolation and clonal propagation of such cells. At the clonal level the stem cells are capable of self regeneration and asymmetrical division. Lineage restriction is demonstrated within developing clones which are sensitive to the local environment. The invention also includes such cells which are transfected with foreign nucleic acid, e.g., to produce an immortalized neural stem cell. The invention further includes transplantation assays which allow for the identification of mammalian multipotent neural stem cells from various tissues and methods for transplanting mammalian neural stem cells and/or neural or glial progenitors into mammals. A novel method for detecting antibodies to neural cell surface markers is disclosed as well as a monoclonal antibody to mouse LNGFR.



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MAMMALIAN MULTIPOTENT NEURAL STEM CELLS

This is a continuation-in-part of U.S. patent application Serial No. 07/996,088, filed October 29, 1992 which is a continuation-in-part of U.S. patent application Serial No. 07/920,617, filed July 27, 1992.

5 Field of the Invention

The invention relates to the isolation, regeneration and use of mammalian multipotent neural stem cells and progeny thereof.

Background

10 The neural crest is a transient embryonic precursor population, whose derivatives include cells having widely different morphologies, characteristics and functions. These derivatives include the neurons and glia of the entire peripheral nervous system,
15 melanocytes, cartilage and connective tissue of the head and neck, stroma of various secretory glands and cells in the outflow tract of the heart (for review, see Anderson, D.J. (1989) Neuron 3:1-12). Much of the knowledge of the developmental potential and fate of
20 neural crest cells comes from studies in avian systems. Fate maps have been established in aves and provide evidence that several different crest cell derivatives may originate from the same position along the neural

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tube (Le Dourain, N.M. (1980) Nature 286:663-669). Schwann cells, melanocytes and sensory and sympathetic neurons can all derive from the truncal region of the neural tube. On the other hand, some derivatives were 5 found to originate from specific regions of the crest, e.g., enteric ganglia from the vagal and sacral regions. These studies also revealed that the developmental potential of the neural crest population at a given location along the neural tube is greater 10 than its developmental fate. This suggests that the new environment encountered by the migrating crest cells influences their developmental fate.

Single-cell lineage analysis *in vivo*, as well as clonal analysis *in vitro*, have reportedly shown that early 15 avian neural crest cells are multipotential during, or shortly after, their detachment and migration from the neural tube. In avian systems, certain clones derived from single neural crest cells in culture were reported to contain both catecholaminergic and pigmented cells 20 (Sieber-Blum, M. et al. (1980) Dev. Biol. 80:96-106). Baroffio, A. et al. (1988) Proc. Natl. Acad. Sci. USA 85:5325-5329, reported that avian neural crest cells from the cephalic region could generate clones which gave rise to highly heterogeneous progeny when grown on 25 growth-arrested fibroblast feeder cell layers.

In vivo demonstration of the multipotency of early neural crest cells was reported in chickens by Bronner-Fraser, M. et al. (1989) Neuron 3:755-766. Individual neural crest cells, prior to their migration from the 30 neural tube, were injected with a fluorescent dye. After 48 hours, the clonal progeny of injected cells were found to reside in many or all of the locations to which neural crest cells migrate, including sensory and sympathetic ganglia, peripheral motor nerves and the 35 skin. Phenotypic analysis of the labelled cells

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revealed that at least some neural crest cells are multipotent *in vivo*.

Following migration from the neural tube, these early multipotent crest cells become segregated into different sublineages, which generate restricted subsets of differentiated derivatives. The mechanisms whereby neural crest cells become restricted to the various sublineages are poorly understood. The fate of neural crest derivatives is known to be controlled in some way by the embryonic location in which their precursors come to reside (Le Douarin, N.M. (1982) The Neural Crest, Cambridge University Press, Cambridge, UK). The mechanism of specification for neural crest cells derivatives is not known. In culture studies described above, investigators reported that clones derived from primary neural crest cells exhibited a mixture of phenotypes (Sieber-Blum, M. et al. (1980) *ibid*; Baroffio, A. et al. (1988) *ibid*; Cohen, A.M. et al. (1975) Dev. Biol. 46:262-280; Dupin, E. et al. (1990) Proc. Natl. Acad. Sci. USA 87:1119-1123). Some clones contained only one differentiated cell type whereas other clones contained many or all of the assayable crest phenotypes.

The observation that apparently committed progenitors and multipotent cells coexist in the neural crest may be interpreted to reflect a pre-existing heterogeneity in the population of primary crest cells or it may reflect asynchrony in a population of cells that undergoes a progressive restriction in developmental potential. Given the uncertainty in the art concerning the developmental potential of neural crest cells, it is apparent that a need exists for the isolation of neural crest cells in clonal cultures. Although culture systems have been established which allow the growth and differentiation of isolated avian neural

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crest cells thereby permitting phenotypic identification of their progeny, culture conditions which allow the self-renewal of multipotent mammalian neural crest cells have not been reported. Such 5 culture conditions are essential for the isolation of mammalian neural crest stem cells. Such stem cells are necessary in order to understand how multipotent neural crest cells become restricted to the various neural crest derivatives. In particular, culture conditions 10 which allow the growth and self-renewal of mammalian neural crest stem cells are desirable so that the particulars of the development of these mammalian stem cells may be ascertained. This is desirable because a number of tumors of neural crest derivatives exist in 15 mammals, particularly humans. Knowledge of mammalian neural crest stem cell development is therefore needed to understand these disorders in humans. Additionally, the ability to isolate and grow mammalian neural crest stem cells *in vitro* allows for the possibility of using 20 said stem cells to treat peripheral neurological disorders in mammals, particularly humans.

Accordingly, it is an object herein to provide clonal cultures of mammalian multipotent neural stem cells and their progeny in feeder cell-independent cultures. 25 Another object of the invention is directed to the demonstration that multipotential stem cells exist in the neural crest. Another object of the invention is the demonstration that these multipotent neural crest stem cells have at least limited self regeneration capacity and undergo lineage restriction in a manner 30 that is sensitive to the local environment.

A further object of the invention is to provide methods which allow the growth and regeneration of multipotent neural stem cells in feeder cell-independent cultures. 35 Another object of the invention is to provide methods

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which allow the differentiation of multipotent neural crest stem cells into at least the progenitors for, as well as, more differentiated neurons and glia of the peripheral nervous system (PNS). A further object of
5 the invention is to provide methods which allow for the identification of mammalian multipotent neural stem cells using transplantation assays. Still further, an object of the invention is to provide methods for transplanting neural crest stem cells or their progeny
10 into a mammal.

A further object of the invention is to extend the above methods to provide clonal cultures of mammalian neural crest stem cells and their progeny, to the detection or purification of glial or neuronal
15 progenitor cells, and to provide methods which allow the growth, regeneration and differentiation of such cells from tissues other than the embryonic neuronal crest. Still further, it is an object herein to provide methods for transplanting progenitors of such
20 glial and neuronal cells and multipotent stem cell precursor thereof into a mammal.

A further object of the invention is to provide cultures of genetically-engineered multipotent neural stem cells and their progeny. Still further, an object
25 of the invention is to provide methods for the generation of cultures of such genetically-engineered multipotent neural stem cells and their progeny including methods for immortalizing such cells.

Further, an object of the invention is to provide
30 monoclonal antibodies capable of recognizing surface markers which characterize multipotent neural stem cells and/or their progeny. A further object is to provide a novel procedure for screening sera and hybridomas for such antibodies.

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Summary of the Invention

In accordance with the foregoing objects, the invention includes the isolation, clonal expansion and differentiation of mammalian multipotent neural stem cells such as those derived from the neural crest. The methods employ novel separation and culturing regimens and bioassays for establishing the generation of multipotent neural stem cells and their derivatives.

These methods result in the production of non-transformed neural stem cells and their progeny. The invention demonstrates, at the clonal level, the self regeneration and asymmetrical division of mammalian neural stem cells for the first time in feeder cell-independent cultures. Lineage restriction is demonstrated within a developing clone and is shown to be sensitive to the local environment. For example, neural crest stem cells cultured on a mixed substrate of poly-D-lysine and fibronectin generate PNS neurons and glia, but on fibronectin alone the stem cells generate PNS glia but not neurons. The neurogenic potential of the neural crest stem cells, while not expressed, is maintained over time on fibronectin. Therefore, both the overt differentiation and maintenance of a latent developmental potential of neural crest stem cells are shown to be sensitive to the environment. The invention further includes transplantation assays which allow for the identification of mammalian multipotent neural stem cells from various tissues. It also includes methods for transplanting mammalian neural stem cells and/or neural or glial progenitors into mammals.

The invention also provides methods for obtaining a cellular composition from mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neural progenitor cell or a multipotent stem cell precursor of such cells. The

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- method comprises preparing a suspension comprising a population of cells from a mammalian tissue; contacting the cell suspension with a culture medium and substrate which permits self-renewal of one or more of the glial or neural progenitor cells or multipotent stem cell precursor, if present, in the cell suspension; and identifying one or more such cells by its ability to self-renew and differentiate feeder-cell independent culture.
- 10 The invention also includes alternate methods for obtaining a cellular composition comprising one or more cells having at least one property characteristic of a glial or neural progenitor cell or a multipotent stem cell precursor thereof. The method comprises preparing
- 15 a suspension comprising cells from a mammalian tissue; contacting the suspension with an antibody capable of forming a complex with a neural cell-specific surface marker on said glial or neural progenitor cells or multipotent stem cell precursor; and isolating the
- 20 complex, if formed, to obtain said cellular composition.

The invention is also directed to cells made according to any of the foregoing methods.

25 The invention also includes cultures of genetically-engineered mammalian multipotent neural stem cells and their progeny. Nucleic acid sequences encoding genes of interest are introduced into multipotent neural stem cells where they are expressed. These genes can include neurotrophic or survival factors and

30 immortalizing oncogenes. In addition, marker genes, such as the *E. coli* β -galactosidase gene, can be introduced to provide neural stem cells and their progeny which can be identified based on the expression of the marker gene. Selectable marker genes, such as

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the neomycin phosphoribosyltransferase (neomycin-resistance, neo') or hisD genes, may be introduced to provide for a population of genetically-engineered stem cells which are identified by the ability to grow in
5 the presence of selective pressure (i.e., medium containing neomycin or L-histidinol). Neural stem cells may be transfected (genetically-engineered) with both a selectable marker and a non-selectable marker to provide neural stem cells which express both gene
10 products.

The invention also includes methods for producing cultures of genetically-engineered mammalian multipotent neural stem cells and their progeny.

Still further, the invention includes methods for
15 immortalizing such cell lines by transfecting a glial or neural progenitor cell or multipotent stem cell precursor thereof with a vector comprising at least one immortalizing gene.

Further, the invention includes monoclonal antibodies
20 capable of recognizing surface markers characteristic of mammalian multipotent neural stem cells and their progeny. The invention also includes a method for screening hybridoma producing such monoclonal antibodies which comprises contacting live neural cells
25 with monoclonal antibodies from a hybridoma and detecting whether the monoclonal antibody binds to the neural cell.

Brief Description of the Drawings

Figure 1A depicts the migration of rat neural crest
30 cells from the neural tube.

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Figure 1B demonstrates the expression of LNGFR and nestin by neural crest cells.

Figures 1C and 1D show the FACS profile from neural crest cells stained with anti-LNGFR (1D) and a control 5 showing the background staining of the secondary antibody (1C).

Figure 2 demonstrates the clonal expansion of LNGFR⁺, nestin⁺ rat neural crest cells.

Figure 3 is a flow chart summarizing experiments 10 demonstrating the multipotency of mammalian neural crest cells.

Figure 4 demonstrates the expression of neuronal traits in clones derived from LNGFR⁺ founder cells.

Figure 5 demonstrates the expression of Schwann cell 15 phenotype by neural crest-derived glia.

Figure 6 shows the expression of peripherin, GFAP, and O₄ in a clone derived from a LNGFR⁺ founder cell.

Figure 7 is a flow chart summarizing experiments 20 demonstrating the self-renewal of mammalian neural crest cells.

Figure 8 demonstrates the self-renewal of multipotent neural crest cells.

Figure 9 demonstrates the multipotency of secondary founder cells.

25 Figure 10 provides a flow chart summarizing experiments demonstrating the substrate effect on the fate of mammalian neural crest cells.

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Figure 11 demonstrates that the neuronal differentiation of multipotent neural crest cells is affected by their substrate.

5 Figure 12 summarizes the percentage of different clone types which result when founder cells are grown on either FN or FN/PDL substrates.

Figure 13 provides a flow chart summarizing experiments demonstrating the instructive effect of the substrate on neural crest cell fate.

10 Figure 14 summarizes the percentage of the different clone types which result when founder cells are treated with a PDL lysine overlay at 48 hours (panel A) or day 5 (panel B).

15 Figure 15 demonstrates the genetic-engineering of a multipotent neural stem cell. Panel A depicts the expression of *E. coli* β -galactosidase (*lacZ*) in neural crest stem cells following infection with a *lacZ*-containing retrovirus. β -galactosidase⁺ cells are indicated by the solid arrows. Panel B depicts neural 20 crest stem cells in phase contrast, in the same microscopic field as shown in Panel A. Cells which do not express β -galactosidase are indicated by open arrows.

25 Figure 16 demonstrates the specificity of a supernatant from a hybridoma culture producing monoclonal antibody specific to mouse LNGFR. Supernatants were screened using live Schwann cells isolated from mouse sciatic nerve. Panel A shows that most cells are stained with anti-LNGFR antibody (red staining; open arrows). Panel 30 B shows Schwann cell nuclei counter stained with DAPI. Comparison with Panel A reveals a few cells not labeled by anti-LNGFR antibody (blue staining; open arrows).

Detailed Description of the Invention

The invention is directed, in part, to the isolation and clonal propagation of non-transformed mammalian neural crest stem cells and to multipotent neural stem cells from other embryonic and adult tissue. The invention also includes the production of neural crest stem cell and multipotent neural stem cell derivatives including progenitor and more differentiated cells of the neuronal and glial lineages. The invention is illustrated using neural crest stem cells isolated from the rat. The invention, however, encompasses all mammalian neural crest stem cells and multipotent neural stem cells and their derivatives and is not limited to neural crest stem cells from the rat.

Mammalian neural crest stem cells and multipotent neural stem cells and their progeny can be isolated from tissues from human and non-human primates, equines, canines, felines, bovines, porcines, lagomorphs, etc.

The invention encompasses several important methodological innovations: 1) the use of monoclonal antibodies to the low-affinity Nerve Growth Factor Receptor (LNGFR) as a cell surface marker to isolate and identify neural crest stem cells, a method extensible to other neural stem cell populations as well; 2) the development of cell culture substrates and medium compositions which permit the clonal expansion of undifferentiated neural crest cells; 3) the development of culture substrates and medium compositions which permit the differentiation of mammalian neural crest cells into their differentiated derivatives (including but not restricted to peripheral neurons and glia) in clonal culture.

The invention also provides neural crest stem cells and other multipotent neural stem cells. It is important

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to understand that such cells could not be identified as stem cells without the development of the isolation and cell culture methodologies summarized above. The identification of a neural stem cell requires that
5 several criteria be met: 1) that the cell be an undifferentiated cell capable of generating one or more kinds of differentiated derivatives; 2) that the cell have extensive proliferative capacity; 3) that the cell be capable of self-renewal or self-maintenance (Hall et
10 al. (1989) Development 106:619; Potten et al. (1990) Crypt. Development 110:1001). The concept of a stem cell as obligatorily capable of "unlimited" self-renewal is applicable only to regenerating tissues such as skin or intestine. In the case of a developing
15 embryo stem cells may have limited self-renewal capacity but be stem cells nevertheless (Potten et al. (1990) *supra*). The development of clonal culture methods permitted the demonstration of criteria 1 and 2 herein. The development of sub-clonal culture
20 methods (i.e., the ability to clone single neural stem cells, and then re-clone progeny cells derived from the original founder cell) further permitted the demonstration herein of criterion 3.

To appreciate the significance of this demonstration,
25 consider an alternative hypothesis for cells from the neural crest: individual undifferentiated neural crest cells divide to generate both neurons and glia (i.e., meet criteria 1 and 2 above), but the daughter cells produced by these initial cell divisions are committed
30 to producing either neurons or glia, but not both. In this case, the neural crest cell is a progenitor cell but not a stem cell, because it does not have self-renewal capacity. If this were the case, then upon sub-cloning of neural crest cell clones, the resulting
35 "secondary" clones could contain either neurons or glia, but not both. This is not observed. Rather,

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- most or all of the secondary clones contain both neurons and glia, like their parent clones. This experiment thus provides the first definitive evidence that neural progenitor cells from any region of the nervous system have stem cell properties. In no other set of published experiments have these stringent criteria for stem cell properties been met, despite claims that "stem cells" have been isolated or identified (Cattaneo et al. (1991) Trends Neurosci. 14:338; Reynolds et al. (1992) Science 255:1707) from the mammalian central nervous system. This in part reflects imprecise use of the term "stem cell" and in part the failure to perform adequate experimental tests to support the existence of such cells.
- 15 As used herein, the term "non-transformed cells" means cells which are able to grow *in vitro* without the need to immortalize the cells by introduction of a virus or portions of a viral genome containing an oncogene(s) which confers altered growth properties upon cells by virtue of the expression of viral genes within the transformed cells. These viral genes typically have been introduced into cells by means of viral infection or by means of transfection with DNA vectors containing isolated viral genes.
- 25 As used herein, the term "genetically-engineered cell" refers to a cell into which a foreign (i.e., non-naturally occurring) nucleic acid, e.g., DNA, has been introduced. The foreign nucleic acid may be introduced by a variety of techniques, including, but not limited to, calcium-phosphate-mediated transfection, DEAE-mediated transfection, microinjection, retroviral transformation, protoplast fusion and lipofection. The genetically-engineered cell may express the foreign nucleic acid in either a transient or long-term manner.
- 30 In general, transient expression occurs when foreign
- 35

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DNA does not stably integrate into the chromosomal DNA of the transfected cell. In contrast, long-term expression of foreign DNA occurs when the foreign DNA has been stably integrated into the chromosomal DNA of
5 the transfected cell.

As used herein, an "immortalized cell" means a cell which is capable of growing indefinitely in culture due to the introduction of an "immortalizing gene(s)" which confers altered growth properties upon the cell by
10 virtue of expression of the immortalizing gene(s) within the genetically engineered cell. Immortalizing genes can be introduced into cells by means of viral infection or by means of transfection with vectors containing isolated viral nucleic acid encoding one or
15 more oncogenes. Viruses or viral oncogenes are selected which allow for the immortalization but preferably not the transformation of cells. Immortalized cells preferably grow indefinitely in culture but do not cause tumors when introduced into
20 animals.

As used herein, the term "transformed cell" refers to a cell having the properties of 1) the ability to grow indefinitely in culture and 2) causing tumors upon introduction into animals. "Transformation" refers to
25 the generation of a transformed cell.

As used herein, the term "feeder-cell independent culture" or grammatical equivalents means the growth of cells *in vitro* in the absence of a layer of different cells which generally are first plated upon a culture
30 dish to which cells from the tissue of interest are added. The "feeder" cells provide a substratum for the attachment of the cells from the tissue of interest and additionally serve as a source of mitogens and survival factors. The feeder-cell independent cultures herein

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utilize a chemically defined substratum, for example fibronectin (FN) or poly-D-lysine (PDL) and mitogens or survival factors are provided by supplementation of the liquid culture medium with either purified factors or
5 crude extracts from other cells or tissues. Therefore, in feeder-cell independent cultures, the cells in the culture dish are primarily cells derived from the tissue of interest and do not contain other cell types required to support the growth of the cells derived
10 from the tissue of interest.

As used herein, the term "clonal density" means a density sufficiently low enough to result in the isolation of single, non-impinging cells when plated in a culture dish, generally about 225 cells/100 mm
15 culture dish.

As used herein, the term "neural crest stem cell" means a cell derived from the neural crest which is characterized by having the properties (1) of self-renewal and (2) asymmetrical division; that is, one
20 cell divides to produce two different daughter cells with one being self (renewal) and the other being a cell having a more restricted developmental potential, as compared to the parental neural crest stem cell. The foregoing, however, is not to be construed to mean
25 that each cell division of a neural crest stem cell gives rise to an asymmetrical division. It is possible that a division of a neural crest stem cell can result only in self-renewal, in the production of more developmentally restricted progeny only, or in the
30 production of a self-renewed stem cell and a cell having restricted developmental potential.

As used herein, the term "multipotent neural stem cell" refers to a cell having properties similar to that of a neural crest stem cell but which is not necessarily

derived from the neural crest. Rather, as described hereinafter, such multipotent neural stem cells can be derived from various other tissues including neural epithelial tissue from the brain and/or spinal cord of
5 the adult or embryonic central nervous system or neural epithelial tissue which may be present in tissues comprising the peripheral nervous system. In addition, such multipotent neural stem cells may be derived from other tissues such as lung, bone and the like utilizing
10 the methods disclosed herein. It is to be understood that such cells are not limited to multipotent cells but may comprise a pluripotent cell capable of regeneration and differentiation to different types of neurons and glia, e.g., PNS and CNS neurons and glia or
15 progenitors thereof. In this regard, it should be noted that the neural crest stem cells described herein are at least multipotent in that they are capable, under the conditions described, of self-regeneration and differentiation to some but not all types of neurons and glia *in vitro*. Thus, a neural crest stem cell is a multipotent neural stem cell derived from a
20 specific tissue, i.e., the embryonic neural tube.

In most embodiments, neural crest stem cells are further characterized by a neural cell-specific surface marker. Such surface markers in addition to being found on neural crest stem cells may also be found on other multipotent neural stems derived therefrom, e.g., glial and neuronal progenitor cells of the peripheral nervous system (PNS) and central nervous system (CNS).
25 An example is the cell surface expression of a nerve growth factor receptor on neural crest stem cells. In rat, humans and monkeys this nerve growth factor receptor is the low-affinity nerve growth factor receptor (LNGFR). Such stem cells may also be characterized by the expression of nestin, an
30 intracellular intermediate filament protein. Neural
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crest stem cells may be further characterized by the absence of markers associated with mature PNS neuronal or glial cells. In the rat, such markers include sulfatide, glial fibrillary acidic protein (GFAP) and myelin protein P₀ in PNS glial cells and peripherin and neurofilament in PNS neuronal cells.

LNGFR is a receptor for nerve growth factor, a neurotrophic factor shown to be responsible for neuronal survival *in vivo*. LNGFR is found on several mammalian cell types including neural crest cells and Schwann cells (glial cells of the PNS) as well as on the surface of cells in the ventricular zone throughout the embryonic central nervous systems. (See, e.g., Yan et al. (1988) J. Neurosci. 8:3481-3496 and Heuer, J.G et al. (1980) Neuron 5:283-296 which studied such cells in the rat and chick systems, respectively.) Antibodies specific for LNGFR have been identified for LNGFR from rat monoclonal antibodies 217c (Peng, W.W. et al. (1982) Science 215:1102-1104) and 192-Ig (Brockes, J.P. et al. (1977) Nature 266:364-366 and Chandler, C.E. et al. (1984) J. Biol. Chem. 259:6882-6889) and human (Ross, A.H. et al. (1984) Proc. Natl. Acad. Sci. USA 81:6681-6685; Johnson, et al. (1986) Cell 47:545-554; Loy et al. (1990) J. Neurosci. Res. 27:651-644). The monoclonal antibody against human LNGFR has been reported to cross-react with LNGFR from monkeys (Mufson, E.G. et al. (1991) J. Comp. Neurol. 308:555-575). The DNA sequence has been determined for rat and human LNGFR (Radeke, M.J. et al. (1987) Nature 325:593-597 and Chao, M.V. et al. (1986) Science 232:518-521, respectively) and is highly conserved between rat and human.

Using the following techniques, monoclonal antibodies specific for LNGFR from any desired mammalian species are generated by first isolating the nucleic acid

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encoding the LNGFR protein. One protocol for obtaining such nucleic acid sequences uses one or more nucleic acid sequences from a region of the LNGFR gene which is highly conserved between mammalian species, e.g., rat
5 and human, as a hybridization probe to screen a genomic library or a cDNA library derived from mammalian tissue from the desired species (Sambrook, J. et al. (1989) Cold Spring Harbor Laboratory Press. Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 8.3-8.80,
10 9.47-9.58 and 11.45-11.55). The cloned LNGFR sequences are then used to express the LNGFR protein or its extracellular (ligand binding) domain in an expression host from which the LNGFR protein is purified. Purification is performed using standard techniques
15 such as chromatography on gel filtration, ion exchange or affinity resins. The purified LNGFR is then used to immunize an appropriate animal (e.g., mouse, rat, rabbit, hamster) to produce polyclonal antisera and to provide spleen cells for the generation of hybridoma
20 cell lines secreting monoclonal antibodies specific for LNGFR of the desired species (Harlow, E. et al. (1988) Cold Spring Harbor Laboratory Press, Antibodies: A Laboratory Manual, pp. 139-242).

A novel screening method can be used to detect the production of antibody against LNGFR or any other surface marker which characterizes a multipotent neural stem cell or progeny thereof. The method can be practiced to detect animals producing polyclonal antibodies against a particular antigen or to identify
25 and select hybridomas producing monoclonal antibodies against such antigens. In this method, serum from an immunized animal or supernatent from a hybridoma culture is contacted with a live neural cell which displays a surface marker characteristic of a
30 particular neural cell line. Detection of whether binding has occurred or not is readily determined by
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any number of known methods. A particularly preferred method is to use labeled antibody which is specific for the immunoglobulins produced by the species which is immunized with the particular antigen and which is a 5 source for polyclonal serum and spleen cells for hybridoma formation.

The live neural cell used in the foregoing antibody assay is dependent upon the particular surface marker for which an antibody is desired. In the examples, a 10. monoclonal antibody for mouse LNGFR was identified using a dissociated primary culture of Schwann cells. In conjunction with the assay disclosed in the examples, mouse fibroblasts acted as a negative control. However, primary cultures of other cell lines 15 can be used to detect monoclonal antibodies to LNGFR. For example, forebrain cholinergic neurons or sensory neurons can be used. In addition, a primary culture of epithelial cells can be used as a negative control.

Other markers found on neural cells include Platelet 20 Derived Growth Factor Receptor (PDGFR), Fibroblast Growth Factor (FGF) and Stem Cell Factor Receptor (SCFR). Cells useful for detecting monoclonal antibodies to PDGFR and FGF include primary cultures of glial cells or fibroblasts. Negative controls include 25 cultures of epithelial cells and neuroblastomas. SCFR is expressed on a subset of neuronal cells. Primary cultures of melanocytes or melanoma cells can be used to detect monoclonal antibodies to this receptor. Negative controls include primary cultures of 30 fibroblasts and glial cells.

It is not always necessary to generate polyclonal or monoclonal antibodies that are species specific. Monoclonal antibodies against an antigenic determinant from one species may react against that antigen from

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more than one species. For example, as stated above, the antibody directed against the human LNGFR molecule also recognizes LNGFR on monkey cells. When cross-reactive antibodies are available, there is no need to 5 generate antibodies which are species specific using the methods described above.

Nestin, a second marker in the neural crest stem cell, is an intermediate filament protein primarily located intracellularly, which has been shown to be present in 10 CNS neuroepithelial cells and Schwann cells in the peripheral nervous system of rats (Friedman et al. (1990) J. Comp. Neurol. 295:43-51). Monoclonal antibodies specific for rat nestin have been isolated: Rat 401, (Hockfield, S. et al. (1985) J. Neurosci. 15 5(12):3310-3328). A polyclonal rabbit anti-nestin antisera has been reported which recognizes mouse nestin (Reynolds, D.A. et al. (1992) Science 255:1707-1710). The DNA sequences encoding the rat nestin gene have been cloned (Lendahl, U. et al. (1990) Cell 20 60:585-595). These DNA sequences are used to isolate nestin clones from other mammalian species. These DNA sequences are then used to express the nestin protein and monoclonal antibodies directed against various mammalian nestins are generated as described above for 25 LNGFR.

As used herein, the term "glial progenitor cell" refers to a cell which is intermediate between the fully differentiated glial cell and a precursor multipotent neural stem cell from which the fully differentiated 30 glial cell develops. In general, such glial progenitor cells are derived according to the methods described herein for isolating such cells from various tissues including adult and embryonic CNS and PNS tissue as well as other tissues which may potentially contain 35 such progenitors.

As used herein, the term "PNS glial progenitor cell" means a cell which has differentiated from a mammalian neural crest stem cell which is committed to the PNS glial lineage and is a dividing cell but does not yet express surface or intracellular markers found on more differentiated, non-dividing PNS glial cells. Such progenitor cells are preferably obtained from neural crest stem cells isolated from the embryonic neural crest which have undergone further differentiation.

However, equivalent cells may be derived from other tissue. When PNS glial progenitor cells are placed in appropriate culture conditions they differentiate into PNS glia expressing the appropriate differentiation markers, for example, sulfatide and GFAP.

Sulfatide is a glycolipid molecule found on the surface of Schwann cells and oligodendrocytes in rats, mice, chickens and humans. The expression of sulfatide on Schwann cells is dependent upon either axonal contact or exposure to cyclic AMP or analogs thereof, such as forskolin (Mirsky, R. et al. (1990) Development 109:105-116). Monoclonal antibodies specific for sulfatide have been reported (Sommer, I. et al. (1981) Dev. Biol. 83:311-327).

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein specifically expressed by astrocytes and glial cells of the CNS and by Schwann cells, the glial cells of the PNS (Jessen, K.R. et al. (1984) J. Neurocytology 13:923-934 and Fields, K.L. et al. (1989) J. Neuroimmuno. 8:311-330). Monoclonal antibodies specific for GFAP have been reported (Debus et al. (1983) Differentiation 25:193-203). Mouse and human GFAP genes have been cloned (Cowan, N.J. et al. (1985) N.Y. Acad. Sci. 455:575-582 and Bongcamrudlowss, D. et al. (1991) Cancer Res. 51:1553-1560, respectively). These DNA sequences are used to isolate

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GFAP clones from other mammalian species. These DNA sequences are then used to express the GFAP protein and monoclonal antibodies directed against various mammalian GFAPs are generated as described above for
5 LNGFR.

As used herein, the term "factors permissive for PNS glial cell differentiation" means compounds, such as, but not limited to, protein or steroid molecules or substrates such as FN or PDL, which permit at least
10 neural crest stem cells to become restricted to the PNS glial lineage. Such lineage-restricted progeny of neural crest stem cells include glial progenitor cells, which are at least bipotential, in that they can divide to give rise to self, as well as, more mature non-
15 dividing PNS glial cells.

As used herein, the term "neuronal progenitor cell" refers to a cell which is intermediate between the fully differentiated neuronal cell and a precursor multipotent neural stem cell from which the fully
20 differentiated neuronal cell develops. In general, such neuronal progenitor cells are derived according to the methods described herein for isolating such cells from various tissues including adult and embryonic CNS and PNS tissue as well as other tissues which may
25 potentially contain such progenitors.

As used herein, the term "PNS neuronal progenitor cell" means a cell which has differentiated from a mammalian neural crest stem cell which is committed to one or more PNS neuronal lineages and is a dividing cell but
30 does not yet express surface or intracellular markers found on more differentiated, non-dividing PNS neuronal cells. Such progenitor cells are preferably obtained from neural crest stem cells isolated from the embryonic neural crest which have undergone further

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differentiation. However, equivalent cells may be derived from other tissue. When PNS neuronal progenitor cells are placed in appropriate culture conditions they differentiate into mature PNS neurons 5 expressing the appropriate differentiation markers, for example, peripherin, neurofilament and high-polysialic acid neural cell adhesion molecule (high PSA-NCAM).

Peripherin, a 57 kDa intermediate filament protein, is expressed in adult rodents primarily in peripheral 10 neurons. More limited expression of peripherin is found in some motoneurons of the spinal cord and brain stem and a limited group of CNS neurons. Peripherin is expressed in rat embryos primarily in neurons of peripheral ganglia and in a subset of ventral and 15 lateral motoneurons in the spinal cord (Gorham, J.D. et al. (1990) Dev. Brain Res. 57:235-248). Antibodies specific for this marker have been identified in the rat (Portier, M. et al. (1983/84) Dev. Neurosci. 6:335-344). The DNA sequences encoding the rat peripherin 20 gene have been cloned (Thompson, M.A. et al. (1989) Neuron 2:1043-1053). These DNA sequences are used to isolate DNA sequences for the peripherin gene in other mammals that are used to express the protein and generate antibodies directed against other mammalian 25 peripherin proteins, as described above for LNGFR.

Neurofilaments are neuron-specific intermediate filament proteins. Three neurofilament (NF) proteins have been reported: NF68, a 68 kD protein also called 30 NF-L (Light); NF160, a 160 kD protein also called NF-M (Medium); NF200, a 200 kD protein also called NF-H (Heavy). In general, there is coordinate expression of all three NF proteins in neurons. The DNA sequences encoding the rat NF200 and NF160 proteins have been cloned (Dautigny, A. et al. (1988) Biochem. Biophys. Res. Commun. 154:1099-1106 and Napolitano, E.W. et al. 35

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- (1987) J. Neurosci. 7:2590-2599, respectively). All three NF protein genes have been cloned in mice and humans. Mouse NF68 nucleic acid sequences were reported in Lewis, S.A. et al. (1985) J. Cell Biol. 100:843-850.
- 5 Mouse NF160 nucleic acid sequences were reported in Levy, E. et al. (1987) Eur. J. Biochem. 166:71-77. Mouse NF200 nucleic acid sequences were reported in Shneidman, P.S. et al. (1988) Mol. Brain Res. 4:217-231. In humans, nucleic acid sequences were reported
- 10 for: NF68, Julien, J.-P. et al. (1987) Biochem. Biophys. Acta. 909:10-20; NF160, Myers, M.W. et al. (1987) EMBO J. 6:1617-1626; NF200, Lee, J.F. et al. (1988) EMBO J. 7:1947-1955. These DNA sequences are used to produce the protein for the production of
- 15 antibodies or to isolate other mammalian NF genes and the proteins expressed and antibodies generated for any desired species, as described above for LNGFR. As used herein, the term "NF⁺" means expression of one or more of the three NF proteins.
- 20 As used herein, the term "factors permissive for PNS neuronal cell differentiation" means compounds, such as, but not limited to, protein or steroid molecules or substrates such as FN or PDL, which permit at least a neural crest stem cell to become restricted to the PNS neuronal lineage. Such lineage-restricted progeny of neural crest stem cells include PNS neuronal progenitor cells, which are at least bipotential, in that they can divide to give rise to self, as well as, more mature, non-dividing PNS neurons.
- 25
- 30 Mammalian neural crest stem cell compositions are provided which serve as a source for neural crest cell derivatives such as neuronal and glial progenitors of the PNS which in turn are a source of PNS neurons and glia. Methods are provided for the isolation and
- 35 clonal culture of neural crest stem cells, in the

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absence of feeder cells. In the examples provided, these methods utilize a chemically defined medium which is supplemented with chick embryo extract as a source of mitogens and survival factors. Factors present in
5 the extract of chicken embryos allow the growth and self renewal of rat neural crest stem cells. However, media used to isolate and propagate rat neural crest stem cells can be used to isolate and propagate neural crest stem cells from other mammalian species, such as
10 human and non-human primates, equines, felines, canines, bovines, porcines, lagomorphs, etc.

Culture conditions provided herein allow the isolation self-renewal and differentiation of mammalian neural crest stem cells and their progeny. These culture
15 conditions may be modified to provide a means of detecting and evaluating growth factors relevant to mammalian neural crest stem cell self-renewal and the differentiation of the stem cell and its progeny. These modifications include, but are not limited to,
20 changes in the composition of the culture medium and/or the substrate and in the specific markers used to identify either the neural crest stem cell or their differentiated derivatives.

Culture conditions are provided which allow the differentiation of mammalian neural crest stem cells into the PNS neuronal and glial lineages in the absence
25 of feeder cell layers. In addition to liquid culture media, these culture conditions utilize a substratum comprising fibronectin alone or in combination with
30 poly-D-lysine. In the examples provided, human fibronectin is utilized for the culturing of rat neural crest stem cells and their progeny. Human fibronectin can be used for the culturing of neural crest stem cells isolated from avian species as well as from any
35 mammal, as the function of the fibronectin protein is

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highly conserved among different species. Cells of many species have fibronectin receptors which recognize and bind to human fibronectin.

In order to isolate the subject neural crest stem cells, it is necessary to separate the stem cell from other cells in the embryo. Initially, neural crest cells are obtained from mammalian embryos.

For isolation of neural crest cells from mammalian embryos, the region containing the caudal-most 10 somites are dissected from early embryos (equivalent to gestational day 10.5 day in the rat). These trunk sections are transferred in a balanced salt solution to chilled depression slides, typically at 4°C, and treated with collagenase in an appropriate buffer solution such as Howard's Ringer's solution. After the neural tubes are free of somites and notochords, they are plated onto fibronectin (FN)-coated culture dishes to allow the neural crest cells to migrate from the neural tube. Twenty-four hours later, following removal of the tubes with a sharpened tungsten needle, the crest cells are removed from the FN-coated plate by treatment with a Trypsin solution, typically at 0.05%. The suspension of detached cells is then collected by centrifugation and plated at an appropriate density, generally 225 cells/100mm dish in an appropriate chemically defined medium. This medium is preferentially free of serum and contains components which permit the growth and self-renewal of neural crest stem cells. The culture dishes are coated with an appropriate substratum, typically a combination of FN and poly-D-lysine (PDL).

Procedures for the identification of neural crest stem cells include incubating cultures of crest cells for a short period of time, generally 20 minutes, at room

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temperature, generally about 25°C, with saturating levels of antibodies specific for a particular marker, e.g., LNGFR. Excess antibody is removed by rinsing the plate with an appropriate medium, typically L15 medium (Gibco) supplemented with fresh vitamin mix and bovine serum albumin (L-15 Air). The cultures are then incubated at room temperature with a fluorochrome labelled secondary antibody, typically Phycoerythrin R-conjugated secondary antibody (TAGO) at an appropriate dilution for about 20 minutes. Excess secondary antibodies are then removed using an appropriate medium, such as L-15 Air. The plates are then covered with the chemically defined growth medium and examined with a fluorescence microscope. Individual LNGFR⁺ clones are isolated by fluorescence activated cell sorting (FACS) or, more typically, by marking the plate under the identified clone. The markings are typically made to a diameter of 3-4 mm, which generally allows for the unambiguous identification of the progeny of the founder cell at any time during an experiment. If desired, individual LNGFR⁺ clones are removed from the original plate by trypsinization with the use of cloning cylinders.

Procedures for permitting the differentiation of stem cells include the culturing of isolated stem cells in a medium permissive for differentiation to a desired lineage, such as Schwann cell differentiation (SCD) medium. Other procedures include growth of isolated stem cells on substrates capable of permitting differentiation, such as FN or FN and PDL.

Procedures for the serial subcloning of stem cells and their derivatives include the trypsinization of individual clones, as described above, followed by replating the clone on a desired substrate and culturing in a desired medium, such as a chemically

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defined medium suitable for maintenance of stem cells or SCD medium permissive for the differentiation of said neural crest stem cells. Crest cells may be identified following serial subcloning by live-cell 5 labeling with an antibody directed against LNGFR, as described above.

The methods described herein provide the basis of functional assays which allow for the identification and production of cellular compositions of mammalian 10 cells which have properties characteristic of neural crest stem cells, glial or neuronal progenitor cells or multipotent stem cell precursor of such progenitor cells. In order to isolate such cells from tissues other than embryonic neural tubes, it is necessary to 15 separate the progenitor and/or multipotent stem cells from other cells in the tissue. The methods presented in the examples for the isolation of neural crest stem cells from neural tubes can be readily adapted for other tissues by one skilled in the art. First, a 20 single cell suspension is made from the tissue; the method used to make this suspension will vary depending on the tissue utilized. For example, some tissues require mechanical disruption of the tissue while other tissues require digestion with proteolytic enzymes 25 alone or in combination with mechanical disruption in order to create the single cell suspension. Tissues such as blood already exists as a single cell suspension and no further treatment is required to generate a suspension, although hypotonic lysis of red 30 blood cells may be desirable. Once the single cell suspension is generated it may be enriched for cells expressing LNGFR or other neural cell-specific markers on their surface. One protocol for the enrichment for LNGFR⁺ cells is by incubating the cell suspension with 35 antibodies specific for LNGFR and isolating the LNGFR⁺ cells. Enrichment for cells expressing a neural cell-

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specific surface marker is particularly desirable when these cells represent a small percentage (less than 5%) of the starting population. The isolation of cells which have complexed with an antibody for a neural 5 cell-specific surface marker such as is carried out using any physical method for isolating antibody-labeled cells. Such methods include fluorescent-activated cell sorting in which case the cells, in general, are further labeled with a fluorescent 10 secondary antibody that binds the anti-LNGFR antibody, e.g., mouse anti-LNGFR and fluorescein label goat anti-mouse IgG; panning in which case the antibody-labeled cells are incubated on a tissue-culture plate coated 15 with a secondary antibody; Avidin-sepharose chromatography in which the anti-LNGFR antibody is biotinylated prior to incubation with the cell suspension so that the complexed cells can be recovered on an affinity matrix containing avidin (i.e., where the antibody is an antibody conjugate with one of the members of a binding pair); or by use of magnetic beads 20 coated with an appropriate anti-antibody so that the labeled LNGFR-expressing cells can be separated from the unlabeled cells with the use of a magnet. All of the foregoing cell isolation procedures are standard 25 published procedures that have been used previously with other antibodies and other cells.

The use of antibodies specific for neural stem cell-specific surface markers results in the isolation of multipotent neural stem cells from tissues other than 30 embryonic neural tubes. For example, as previously indicated, LNGFR is expressed in cells of the ventricular zone throughout the embryonic central nervous system of the rat and chick. This implies that other mammalian species have a similar pattern of LNGFR 35 expression and studies in human with monoclonal antibodies against the human LNGFR (Loy, et al. (1990)

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J. Neurosci. Res. 27:651-654) are consistent with this expectation. Since cells from the ventricular zone (Cattaneo et al. (1991) Trends Neurosci. 14:338-340; Reynolds et al. (1992) Science 255:1707-1710) are 5 likely to be stem cells (Hall et al. (1989) Development 106:619-633; Potter et al. (1990) Development 110:1001-1020) antibodies to neural cell-specific surface markers should prove useful in isolating multipotent neural stem cells from the central and peripheral 10 nervous systems and from other tissue sources.

Alternatively, or in conjunction with the above immuno-isolation step, the cells are plated at clonal density, generally 225 cells/100 mm dish, in an appropriate chemically defined medium on a suitable substrate as 15 described in the examples for isolation of rat neural crest stem cells. The presence of neural crest-like stem cells (e.g., a multipotent neural stem cell) is confirmed by demonstrating that a single cell can both self-renew and differentiate to members of at least the 20 PNS neuronal and glial lineages utilizing the culture conditions described herein. Other types of multipotent neural stem cells are identified by differentiation to other cell type such as CNS neural or glial cells or their progenitors. Depending upon 25 the source of the tissue used in the foregoing methods, multipotent neural stem cells may not be obtained. Rather, further differentiated cell types such as glial and neuronal progenitor cells may be obtained.

Transplantation assay systems described herein provide 30 the basis of functional assays which allow for the identification of mammalian cells which have properties characteristic of neural crest stem cells, multipotent neural stem cells and/or neuronal or glial progenitor cells. Cells of interest, identified by either the in 35 vivo or in vitro assays described above, are

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transplanted into mammalian hosts using standard surgical procedures. The transplanted cells and their progeny are distinguished from the host cells by the presence of species specific antigens or by the expression of an introduced marker gene. The transplanted cells and their progeny are also stained for markers of mature neurons and glia in order to examine the developmental potential of the transplanted cells. This transplantation assay provides a means to identify neural crest stem cells by their functional properties in addition to the *in vitro* culture assays described above.

Additionally, the transplantation of cells having characteristics of multipotent neural stem cells, neural crest stem cells or progenitors of neuronal or glial cells provides a means to investigate the therapeutic potential of these cells for neurological disorders of the PNS and CNS in animal models. Examples of PNS disorders in mice include the *trembler* and *shiverer* strains. The *trembler* mutation is thought to involve a defect in the structural gene for myelin basic protein (MBP). This mutation maps to the same region of chromosome 11 as does the MBP gene. This mutation results in the defective myelination of axons in the PNS. An analogous disorder is seen in humans, Charcot-Marie-Tooth syndrome, which results in progressive neuropathic muscular atrophy.

The *shiverer* mutation in mice results in a severe myelin deficiency throughout the CNS and a moderate hypo-myelination in the PNS. Severe shivering episodes are seen 12 days after birth. An analogous disorder is seen in humans, Guillain-Barre' disease, which is characterized by an acute febrile polyneuritis.

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Cells having characteristics of multipotent neural stem cells, neural crest stem cells or neuronal or glial progenitors of the PNS or CNS (identified by either *in vitro* or *in vivo* assays) are introduced into a mammal exhibiting a neurological disorder to examine the therapeutic potential of these cells. These cells are preferably isolated from a mammal having similar MHC genotypes or the host mammal is immunosuppressed using drugs such as cyclosporin A. The cells are injected into an area containing various peripheral nerves known to be effected in a particular mammal or into the spinal cord or brain for mammals which show involvement of the CNS. The cells are injected at a range of concentrations to determine the optimal concentration into the desired site. Alternatively, the cells are introduced in a plasma clot or collagen gel to prevent rapid dispersal of cells from the site of injection. The effect of this treatment on the neurological status of the model animal is noted. Desired therapeutic effects in the above mutant mice include the reduction or cessation of seizures or improved movement of lower motor extremities.

There is strong interest in identifying the multipotent neural stem cells such as the neural crest stem cell and defining culture conditions which allow the clonal propagation and differentiation of said stem cells. Having possession of a multipotent neural stem cell or a neural crest stem cell allows for identification of growth factors associated with self regeneration. In addition, there may be as yet undiscovered growth factors associated with (1) with the early steps of restriction of the stem cell to a particular lineage; (2) the prevention of such restriction; and (3) the negative control of the proliferation of the stem cell or its derivatives.

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The multipotent neural stem cell, neural crest stem cell, progeny thereof or immortalized cell lines derived therefrom are useful to: (1) detect and evaluate growth factors relevant to stem cell
5 regeneration; (2) detect and isolate ligands, such as growth factors or drugs, which bind to receptors expressed on the surface of such cells or their differentiated progeny (e.g., Glial Growth Factor (GGF), Heregulin and Neu Differentiation Factor (NDF));
10 (3) provide a source of cells which express or secrete growth factors specific to multipotent neural stem cells; (4) detect and evaluate other growth factors relevant to differentiation of stem cell derivatives, such as neurons and glia; (5) produce various neural
15 stem cell derivatives, including both the progenitors and mature cells of a given lineage and (6) provide a source of cells useful for treating neurological diseases of the PNS and CNS in model animal systems and in humans. The culture conditions used herein allow
20 for the growth and differentiation of stem cells *in vitro* and provide a functional assay whereby mammalian tissues can be assayed for the presence of cells having the characteristics of neural stem cells. The transplantation assay described herein also provides a
25 functional assay whereby mammalian neural stem cells may be identified.

As indicated in the examples, neural crest stem cells have been passaged for at least six-ten generations in culture. Although it may be unnecessary to immortalize
30 those or other multipotent neural stem cell lines or progenitor cell lines obtained by the methods described herein, once a cell line has been obtained it may be immortalized to yield a continuously growing cell line useful for screening trophic or differentiation factors
35 or for developing experimental transplantation therapies in animals. Such immortalization can be

obtained in multipotent neural stem cells or progenitors of glial and neuronal cells by genetic modification of such cells to introduce an immortalizing gene.

- 5 Examples of immortalizing genes include: (1) nuclear oncogenes such as *v-myc*, *N-myc*, T antigen and Ewing's sarcoma oncogene (Fredericksen et al. (1988) Neuron 1:439-448; Bartlett, P. et al. (1988) Proc. Natl. Acad. Sci. USA 85:3255-3259, and Snyder, E.Y. et al. (1992) Cell 68:33-51), (2) cytoplasmic oncogenes such as *bcr-abl* and neurofibromin (Solomon, E. et al. (1991) Science 254:1153-1160), (3) membrane oncogenes such as *neu* and *ret* (Aaronson, A.S.A (1991) Science 254:1153-1161), (4) tumor suppressor genes such as mutant p53 and mutant Rb (retinoblastoma) (Weinberg, R.A. (1991) Science 254:1138-1146), and (5) other immortalizing genes such as Notch dominant negative (Coffman, C.R. et al. (1993) Cell 23:659-671). Particularly preferred oncogenes include *v-myc* and the SV40 T antigen.
- 20 Foreign (heterologous) nucleic acid may be introduced or transfected into multipotent neural stem cells or their progeny. A multipotent neural stem cell or its progeny which harbors foreign DNA is said to be a genetically-engineered cell. The foreign DNA may be introduced using a variety of techniques. In a preferred embodiment, foreign DNA is introduced into multipotent neural stem cells using the technique of retroviral transfection. Recombinant retroviruses harboring the gene(s) of interest are used to introduce marker genes, such as the *E. coli* β -galactosidase (*lacZ*) gene, or oncogenes. The recombinant retroviruses are produced in packaging cell lines to produce culture supernatants having a high titer of virus particles (generally 10^5 to 10^6 pfu/ml). The recombinant viral particles are used to infect cultures
- 25
- 30
- 35

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of the neural stem cells or their progeny by incubating the cell cultures with medium containing the viral particles and 8 μ g/ml polybrene for three hours. Following retroviral infection, the cells are rinsed
5 and cultured in standard medium. The infected cells are then analyzed for the uptake and expression of the foreign DNA. The cells may be subjected to selective conditions which select for cells that have taken up and expressed a selectable marker gene.

- 10 In another preferred embodiment, the foreign DNA is introduced using the technique of calcium-phosphate-mediated transfection. A calcium-phosphate precipitate containing DNA encoding the gene(s) of interest is prepared using the technique of Wigler et al. (1979)
15 Proc. Natl. Acad. Sci. USA 76:1373-1376. Cultures of the neural stem cells or their progeny are established in tissue culture dishes. Twenty four hours after plating the cells, the calcium phosphate precipitate containing approximately 20 μ g/ml of the foreign DNA is
20 added. The cells are incubated at room temperature for 20 minutes. Tissue culture medium containing 30 μ M chloroquine is added and the cells are incubated overnight at 37°C. Following transfection, the cells are analyzed for the uptake and expression of the
25 foreign DNA. The cells may be subjected to selection conditions which select for cells that have taken up and expressed a selectable marker gene.

The following is presented by way of example and is not to be construed as a limitation on the scope of the
30 invention. Further, all references referred to herein are expressly incorporated by reference.

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EXAMPLE 1Preparation of Neural Crest Cells

For a given preparation 5-10 timed pregnant female Sprague-Dawley rats (Simonson Laboratories, Gilroy, California) were killed by CO₂ asphyxiation. Embryos were removed and placed into Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, New York) at 4°C for 2-4 hours. Under a dissecting microscope, at room temperature, a block of tissue from a region corresponding to approximately the caudal most 10 somites was dissected from each embryo using an L-shaped electrolytically sharpened tungsten needle. Trunk sections were transferred in HBSS into one well of a 3 well depression slide that had been chilled to 4°C. Trunk sections were treated with collagenase (152 units/mg) (Worthington Biochemical, Freehold, New Jersey) made to a concentration of 0.75 mg/ml in Howard's Ringer's solution (per 1 liter of dH₂O: NaCl 7.2g; CaCl₂ 0.17g; KCl 0.37g) and sterilized, by passage through a 0.22 µm filter prior to use. The collagenase solution was exchanged at least 3 times and with each exchange the trunk sections were vigorously triturated by passage through a pasteur pipet. After incubation at 37°C for 20 minutes in humidified CO₂ atmosphere, the trunk sections were triturated very gently until most of the neural tubes were free and clean of somites and notochords. The collagenase solution was quenched by repeated exchanges with cold complete medium (described below). The neural tubes were plated onto fibronectin-coated (substrate preparation is described below) 60mm tissue culture dishes (Corning, Corning, New York) that had been rinsed with complete medium. After a 30 minute incubation to allow the neural tubes to attach, dishes were flooded with 5 ml of medium. After a 24 hour

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culture period, using an L-shaped electrolytically sharpened tungsten needle and an inverted phase contrast microscope equipped with a 4X objective lens, each neural tube was carefully scraped away from the 5 neural crest cells that had migrated onto the substrate. Crest cells were removed by a 2 minute 37°C treatment with 0.05% Trypsin solution (Gibco). The cells were centrifuged for 4 minutes at 2000 r.p.m. and the pellet was resuspended into 1 ml of fresh complete 10 medium. Typically the cells were plated at a density of 225 cells/100 mm dish.

Substrate Preparation

A. Fibronectin (FN) Substrate

Tissue culture dishes were coated with human plasma fibronectin (New York Blood Center, New York, New York) 15 in the following way. Lyophilized fibronectin was resuspended in sterile distilled water (dH₂O) to a concentration of 10 mg/ml and stored at -80°C until used. The fibronectin stock was diluted to a 20 concentration of 250 mg/ml in Dulbecco's phosphate buffered saline (D-PBS) (Gibco). The fibronectin solution was then applied to tissue culture dishes and immediately withdrawn.

B. Poly-D-Lysine (PDL) and FN Substrate

Sterile poly-D-Lysine (PDL) was dissolved in dH₂O to a 25 concentration of 0.5 mg/ml. The PDL solution was applied to tissue culture plates and immediately withdrawn. The plates were allowed to dry at room temperature, rinsed with 5 ml of dH₂O and allowed to 30 dry again. Fibronectin was then applied, as described above, over the PDL.

EXAMPLE 2

Development of a Defined Medium for
the Growth of Rat Neural Crest Stem Cells

A serum-free, chemically defined basal medium was
5 developed based on the formulations of several existing
defined media. This basal medium consists of L15-CO₂
formulated as described by Hawrot, E. et al. (1979)
Methods in Enzymology 58:574-583 supplemented with
additives described by Bottenstein, J.E. et al. (1979)
10 Proc. Natl. Acad. Sci. USA 76:514-517 and further
supplemented with the additives described by Sieber-
Blum, M. et al. (1985) Exp. Cell Res. 158:267-272. The
final recipe is given here: to L15-CO₂ add, 100 µg/ml
15 transferrin (Calbiochem, San Diego, California), 5
µg/ml insulin (Sigma, St. Louis, MO), 16 µg/ml
putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM
selenious acid (Sigma), 1 mg/ml bovine serum albumin,
crystallized (Gibco), 39 pg/ml dexamethasone (Sigma),
35 ng/ml retinoic acid (Sigma), 5 µg/ml α-d, 1-
20 tocopherol (Sigma), 63 µg/ml p-hydroxybuyrate (Sigma),
25 ng/ml cobalt chloride (Sigma), 1 µg/ml biotin
(Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml
glycerol, 100 ng/ml α-melanocyte stimulating hormone
(Sigma), 10 ng/ml prostaglandin E1 (Sigma), 67.5 ng/ml
25 triiodothyronine (Aldrich Chemical Company, Milwaukee,
Wisconsin), 100 ng/ml epidermal growth factor (Upstate
Biotechnology, Inc., Lake Placid, New York), 4 ng/ml
bFGF (UBI), and 20 ng/ml 2.55 NGF (UBI).

To allow the growth and regeneration of neural crest
30 stem cells in feeder cell-independent cultures, it was
necessary to supplement the basal medium with 10% chick
embryo extract (CEE). This supplemented medium is
termed complete medium.

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CEE is prepared as follows: chicken eggs were incubated for 11 days at 38°C in a humidified atmosphere. Eggs were washed and the embryos were removed, and placed into a petri dish containing sterile Minimal Essential
5 Medium (MEM with Glutamine and Earle's salts) (Gibco) at 4°C. Approximately 10 embryos each were macerated by passage through a 30 ml syringe into a 50 ml test tube (Corning). This typically produced 25 ml of volume. To each 25 ml was added 25 ml of MEM. The
10 tubes were rocked at 4°C for 1 hour. Sterile hyaluronidase (1 mg/25 g of embryo) (Sigma) was added and the mixture was centrifuged for 6 hours at 30,000 g. The supernatant was collected, passed first through a 0.45 µm filter, then through a 0.22 µm filter and
15 stored at -80°C until used.

At the low cell densities necessary for survival and proliferation of individual neural crest cells, either fetal calf serum (FCS, JR Scientific) or CEE was required, in addition to the basal medium, for clone
20 formation. When FCS was used to supplement the medium, it was heat inactivated by treatment at 55°C for 30 minutes. FCS was stored at -20°C and passed through a 0.22 µm filter prior to use.

CEE is preferred as a supplement, as in the presence of
25 FCS, most of the cells derived from the neural crest exhibit a flattened, fibroblastic morphology and expression of LNGFR is extinguished. In the absence of both FCS and CEE, clone formation from neural crest cells was greatly attenuated.

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EXAMPLE 3

Isolation and Cloning of
Multipotent Rat Neural Crest Cells

5 A. Identification of Antibody Markers
Expressed by Neural Crest Cells

In order to identify and isolate rat neural crest cells, it was necessary to identify antibody markers that could be used to recognize these cells. When E10.5 neural tubes were explanted onto a fibronectin (FN) substratum, many of the neural crest cells that emigrated from the neural tubes over the next 24 hours expressed the low-affinity NGF receptor (LNGFR), recognized by monoclonal antibodies 192-Ig and 217c. The outgrowth of neural crest cells from the dorsal side of the explanted neural tube following 24 hours growth in culture is shown in Figure 1, panel A. Figure 1, panel B shows the expression of LNGFR (green fluorescence) and nestin (red fluorescence) in neural crest cells.

20 Neural crest cells were labeled with antibodies as follows: For cell surface antigens, such as LNGFR, it was possible to label the living cells in culture. The cultures were incubated with primary antibody solution for 20 minutes at room temperature. The cultures were 25 washed twice with L15 medium (Gibco) supplemented with 1:1:2, fresh vitamin mix (FVM) (Hawrot, E. et al. (1979), *ibid*), and 1 mg/ml bovine serum albumin (L15 Air). The cultures were then incubated for 20 minutes at room temperature with Phycoerythrin R conjugated 30 secondary antibody (TAGO) at a dilution of 1:200 in L-15 Air. The cultures were then rinsed twice with L-15 Air and placed back in their original medium and examined with a fluorescence microscope. Rabbit anti-LNGFR antiserum (Weskamp, G. et al. (1991) Neuron

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6:649-663) was a kind gift of Gisela Weskamp, University of California, San Francisco and was used at a 1:1000 dilution. Monoclonal anti-NCAM antibody 5A5 (Dodd, J. et al. (1988) Neuron 1:105-116) and 5 monoclonal anti-sulfatide antibody O₄ (Sommer, I. et al. (1981) Dev. Biol. 83:311-327) were obtained as hybridoma cells from the Developmental Studies Hybridoma Bank (Johns Hopkins University, Baltimore, Maryland) and prepared as described by the provider.

10 In order to label cells with antibodies directed against intracellular proteins, it was necessary to fix and permeabilize the cells prior to labeling. For most of the immunocytochemistry, formaldehyde fixation was done. Formaldehyde solution 37% was diluted 1:10 into 15 S-MEM with 1mM HEPES buffer (Gibco). Culture were treated for 10 minutes at room temperature with the 3.7% formaldehyde solution and then rinsed 3 times with D-PBS (Gibco).

20 For some intermediate filament proteins (NF and GFAP) formaldehyde fixation was not possible. Cultures were fixed by treatment with a solution of 95% ethanol and 5% glacial acetic acid at -20°C for 20 minutes.

25 For the staining of cytoplasmic antigens, fixed cells were first treated with a blocking solution comprising D-PBS, 0.1% Tween-20 (Bio-Rad Laboratories, Richmond, California) and 10% heat inactivated normal goat serum (NGS) for 15 minutes at room temperature. Primary 30 antibodies were diluted with a solution of D-PBS, 0.1% Tween-20 and 5% NGS. The fixed cells were incubated overnight at 4°C in primary antibody solution then rinsed twice with DPBS, 0.05% Tween-20. Fluorescent secondary antibodies were diluted with D-PBS, 1% NGS and applied to cells for 1 hour at room temperature. The cells were rinsed twice with D-PBS, 0.05% Tween-20.

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To prevent photobleaching, a solution of 8 mg/ml N-propyl gallate in glycerol was placed over the stained cells prior to fluorescence microscopy.

5 Mouse monoclonal anti-GFAP, G-A-5 (Debus et al. (1983) Differentiation 25:193-203) was purchased from Sigma and used at a 1:100 dilution. Mouse monoclonal anti-

NF200, SMI39 was purchased from Sternberger Monoclonals Inc., Baltimore, Maryland and used at a 1:100 dilution. SMI39 reactivity is equivalent to the 06-53 monoclonal antibody described by Sternberger, L.A. et al. (1983) Proc. Natl. Acad. Sci. USA 80:6126-6130. Purified rabbit antibodies to peripherin (preparation 199-6) was obtained from Dr. Linda Parysek, University of Cincinnati, Ohio and was used at a dilution of 1:500.

15 Flow-cytometric analysis indicated that greater than 70% of the neural crest cells show some LNGFR immunoreactivity (Figure 1, panel D). Approximately 25% of the neural crest cells expressed high levels of LNGFR.

20 In some experiments, neural crest cells expressing high levels of LNGFR were further purified by labeling with 192-Ig (anti-LNGFR) and fluorescence-activated cell sorting (FACS). For single cell analysis, however, it proved more convenient to plate the bulk neural crest cell population at clonal density, and then subsequently identify LNGFR-positive cells by live cell-labeling with 192-Ig.

25 Most or all of the neural crest cells also expressed nestin, an intermediate filament protein found in CNS neuroepithelial cells. An individual neural crest cell co-expressing both nestin and LNGFR is shown in Figure 2, panels A-C. Panel A shows the individual neural crest cell in phase contrast. Panels B and C show this cell following staining with both anti-LNGFR (panel B) and anti-nestin (panel C). Figure 2, panels D-F show

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that the clonal progeny of this nestin⁺, LNGFR⁺ neural crest cell also co-express nestin and LNGFR.

B. Cloning of Multipotent Neural Crest Cells

To define the developmental potential of individual neural crest cells, conditions were established that permit the growth of these cells in clonal culture. Figure 3 provides a flow chart depicting the following cell cloning experiments. In Figure 3, plating medium refers to the complete medium, described above and differentiation medium refers to SCD medium, described below. Using an FCS-free, CEE-containing medium (complete or plating medium), single neural crest cells (Figure 4, panel A, phase contrast and panel B, LNGFR staining) were plated on a FN/PDL substratum and allowed to proliferate and differentiate. After 9-14 days, many of the clones founded by single neural crest cells were large and contained cells with a neuronal morphology (Figure 4, panel C, phase contrast). Quantification indicated that > 60% of the clones contained a mixture of neuronal and non-neuronal cells (see below). These neuronal cells could be labeled by antibodies to pan-neuronal markers such as neurofilament (Figure 4, panel E, anti-NF160 staining) and high-polysialic acid (PSA) NCAM (Figure 4, panel D, anti-NCAM staining), as well as by an antibody to peripherin, an intermediate filament protein that is preferentially expressed by peripheral nervous system (PNS) neurons (Figure 4, panel F). Importantly, these neurons did not express either nestin or LNGFR, indicating that they have lost the two markers that characterize the undifferentiated neural crest cell.

The neuron-containing clones also contained non-neuronal cells. These cells continued to express LNGFR and nestin, in contrast to the neurons, and displayed an elongated morphology characteristic of Schwann

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cells. While immature Schwann cells are known to express both LNGFR and nestin, these markers are insufficient to identify Schwann cells in this system since they are expressed by the neural crest precursor 5 cell as well. Expression of more definitive Schwann cell markers was elicited by transferring the cells into a medium known to enhance Schwann cell differentiation. This medium, called Schwann cell differentiation (SCD) medium, contained both 10% FCS 10 and 5 μ M forskolin, an activator of adenylate cyclase.

Figure 5 shows the expression of a Schwann cell phenotype by neural crest-derived glia. Clones plated initially on FN were allowed to grow for a week in complete medium, then transferred into SCD medium and 15 allowed to grow for another 1-2 weeks prior to fixation and immunocytochemistry. Cells of two morphologies, one elongated and the other flattened can be seen in phase contrast (Panels A and D). To demonstrate concordant expression of three markers, LNGFR, O₄ and 20 GFAP, two different double-labeling experiments were performed. Living cells were surface-labeled with monoclonal anti-LNGFR 192IgG (Panel B) and monoclonal O₄ IgM (Panel C) and postfixed. In parallel, other 25 cells from the same clone were first surface-labeled with O₄ and then fixed with acid-ethanol, permeabilized and stained with anti-GFAP (IgG). Note that LNGFR⁺ cells (Panel B) are O₄⁺ and that most or all of the O₄⁺ cells are also GFAP⁺ (Panels E and F). The quality of the O₄ staining in (Panel E) appears different from 30 that in (Panel C) because a redistribution of the antigen occurs following acid-ethanol fixation. In Panel C, the flattened O₄⁺ cells are more weakly stained for LNGFR (Panel B). Such flattening is indicative of myelination, and is consistent with the

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fact that Schwann cells undergoing myelination down-regulate LNGFR and up-regulate O₄.

Following 5-10 days in SCD medium, most or all of the non-neuronal cells in the clones expressed glial fibrillary acidic protein (GFAP), an intermediate filament specific to glial cells, and sulfatide, a cell-surface glycolipid recognized by the monoclonal antibody O₄. Triple-labeling of such "mature" clones with polyclonal anti-peripherin and monoclonal O₄ and anti-GFAP antibodies revealed that sulfatide and GFAP were not expressed by the peripherin-positive neurons and that these two glial markers were coincident in the non-neuronal cell population (Figure 6). Figure 6 shows a clone from a single founder cell in phase contrast (Panel A) which expresses LNGFR (Panel B). This clone was allowed to proliferate and differentiate in complete medium (containing CEE and lacking serum) and then transferred into SCD medium (containing serum and forskol in). After approximately 10 days, the culture was fixed and triple-labeled with rabbit anti-peripherin (Panels C and D, in green/yellow), anti-GFAP (IgG) (Panel C, in red) and O₄ (IgM) (Panel D, blue). Panels C and D are two separate fields from the same clone.

Although GFAP is expressed by astrocytes and sulfatide is expressed by oligodendrocytes in the CNS, the co-expression of these two markers in the same cell is unique to peripheral glial cells (Jessen, K.R. et al. (1990) Devel. 109:91-103 and Mirsky, R. et al. (1990) Devel. 109:105-116).

Therefore, these data indicate that single neural crest cells expressing nestin and LNGFR are able to give rise to clones of differentiated cells containing both peripheral neurons and glia. Differentiation to the

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neuronal phenotype involves both the loss of LNGFR and nestin expression, and the gain of neuronal markers such as neurofilament, high PSA-NCAM and peripherin. On the other hand, in the glial lineage LNGFR and nestin expression persist, and additional glial markers (GFAP and O₄) are acquired. All clones that produced neurons and glia also produced at least one other cell type that did not express any of the differentiation markers tested; the identity of these cells is unknown. Taken together, these data establish the multipotency of the rat neural crest cell identified and isolated by virtue of co-expression of LNGFR and nestin.

EXAMPLE 4

Self-renewal of Multipotent Neural Crest Cells *in vitro*

After 10 days in culture in medium supplemented with 10% CEE and on a FN/PDL substrate, all of the neural crest cell clones that contained neurons also contained non-neuronal cells expressing LNGFR and nestin (as described above). In order to determine whether these cells were immature glia, or multipotent neural crest cells that had undergone self-renewal, serial subcloning experiments were performed. Figure 7 provides a flow chart summarizing these serial subcloning experiments. In Figure 7, "plating medium" refers to complete medium containing CEE and lacking FCS and "differentiation medium" refers to SCD medium containing FCS and forskolin.

For serial sub-cloning experiments, clones were harvested and replated as follows. The primary clones were examined microscopically to ensure that there were no impinging colonies and that the whole clone fits

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within the inscribed circle. Using sterile technique throughout the procedure, glass cloning cylinders (3mm id.) were coated on one end with silicone grease (Dow Corning) and placed about the primary clone so that the 5 grease formed a seal through which medium could not pass. The cells were removed from the cylinder by first treating them with 100 ml of 0.05% Trypsin solution (Gibco) for 3 minutes at 37°C in a humidified 10 5% CO₂ incubator. At room temperature 70 μl of the trypsin solution was removed and replaced with 70 μl of complete medium. The cells were resuspended into the 100 μl volume by vigorous trituration through a pipet tip and the whole volume was diluted into 5 ml of complete medium. The 5 ml was then plated onto 1 or 2 15 60mm dishes which were placed in a humidified 5% CO₂ incubator for 2 hours at which time the medium was exchanged for fresh complete medium. Single founders cells were then identified and allowed to grow into secondary clones as described below.

20 Primary clones founded by LNGFR-positive progenitor cells were allowed to grow for 6 days (Figure 8, Panel A) on a PDL/FN substrate. At this time, clones containing LNGFR-positive cells were identified by live cell surface labeling, and these clones were then 25 removed from their original plates by trypsinization, as described above. The dissociated cells were then replated at clonal density under the same culture conditions as their founder cells. Individual secondary founder cells were identified by labeling live cells 30 with 192-Ig and their positions marked (Figure 8, Panels B and B' show two individual secondary founder cells; Panels C and C' show the clonal progeny of these individual cells at day 17). Both non-neuronal, neurite bearing cells are visible in the clones (Figure 35 8, panels C and C').

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A clone derived from secondary founder cells, such as that shown in Figure 8, was transferred into SCD medium to allow the expression of Schwann cell markers. After approximately 10 days, the subclone was fixed, and 5 double-labeled for NF160 and GFAP (Figure 9, Panel A shows the clone in phase contrast; Panel B shows labeling with anti-NF160; Panel C shows labeling with anti-GFAP). The apparent labeling of neurons in panel C is an artifact due to bleed-through into the 10 fluorescein channel of the Texas Red fluorochrome used on the goat anti-rabbit secondary antibody in panel B.

Additionally, following 10 days of secondary culture, living subclones were scored visually for the presence of neurons and glia by double labeling with 192-Ig 15 (anti-LNGFR) and 5A5, a monoclonal antibody to high PSANCAM.

Single neural crest cells isolated from primary clones were able to proliferate and generate clones containing both neurons and non-neuronal cells, probably glia. 20 Quantitative analysis of clones derived from 16 different primary and 151 secondary founders after ten days in plating medium indicated that over 30% of the total secondary founder cells gave rise to clones containing neurons (N), glia (G) and other (O) cells 25 (Table I, N+G+O). Of the remaining 70% of the founder cells, however, almost 50% failed to form clones and died; thus of the clonogenic (*i.e.*, surviving) founders, 54% were of the N+G+O type (Table I). To confirm that these mixed clones indeed contained glia 30 or glial progenitors, they were transferred to SCD medium and allowed to develop for an additional 7 days, then fixed and double-stained for neurofilament and GFAP expression. As was the case for the primary clones, this treatment caused expression of GFAP in a 35 high proportion of non-neuronal cells in the clones

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(Figure 9), confirming the presence of glia. These data indicate that primary neural crest cells are able to give rise at high frequency to progeny cells retaining the multipotency of their progenitors, 5 indicative of self renewal. However, in several cases secondary clones containing only neurons were found (Table I, N only), and many of the secondary clones contained glia and other cells but not neurons (Table I, G+O). This observation suggests that in addition to 10 self-renewal, proliferating neural crest cells may undergo lineage restriction in vitro as well to give rise to glial or neuronal progenitor cells which are characterized by the capacity to divide and self-renew but are restricted to either the neuronal or glial 15 lineage.

EXAMPLE 5

Substrate Composition Influences the Developmental Fate of Multipotent Neural Crest Cells

The foregoing experiments indicate that neural crest 20 cells grown on a PDL/FN substrate generate clones containing both peripheral neurons and glia. When the same cell population is grown at clonal density on a substrate containing FN only, the resulting clones contain glia and "other" cells but never neurons 25 (Figures 10 and 11, Panels D,E,F). Figure 10 provides a flow chart summarizing the following experiments which demonstrate the substrate effect on the fate of mammalian neural crest cells. Figure 11 shows the immunoreactivity of cells stained for various markers.

30 On FN alone, G+O clones are obtained containing non-neuronal cells expressing high levels of LNGFR immunoreactivity, but neither NCAM⁺ nor neurite-bearing

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cells (Figure 11, panels E,F). By contrast on PDL/FN, the clones contain both LNGFR⁺, NCAM⁻ non-neuronal crest cells and LNGFR⁻, NCAM⁺ neurons (Figure 11, panels B,C). Quantification indicated that on FN alone, 70-80% of
5 the clones are of the G+O phenotype and none of the N+G+O phenotype (Figure 12, panel A), whereas on PDL/FN 60% of the clones are of the N+C+O and only 20% are of the GAO phenotype (Figure 12, panel B). These data indicate that the composition of the substrate affects
10 the phenotype of neural crest cells that develop in culture.

To rule out the possibility that the foregoing results could be explained simply by the failure of neurogenic crest cells to adhere and survive on a FN substrate, a different experiment was performed in which all the
15 crest cells were initially cloned on a FN substrate. Figure 13 provides a flow chart summarizing these experiments. These experiments were performed to demonstrate that differences in attachment and/or survival do not account for differences in eventual
20 clone composition. Subsequently, one group of cells was exposed to PDL as an overlay in liquid media (0.05 mg/ml) after 48 hrs, while a sister culture was retained on FN alone as a control (Figure 13). Clones expressing LNGFR were identified by live cell surface labeling at the time of the PDL overlay and the development of only LNGFR⁺ clones was further monitored.
25 After two weeks, the cultures were transferred to SCD medium for an additional 10 days of culture, and their phenotypes then scored as previously described.
30

By contrast to clones maintained on FN, where no neurons developed, many of the clones exposed to a PDL overlay contained neurons at the end of the culture period (Figure 14, panel A). Moreover, virtually none
35

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of the clones were of the G+O phenotype after the PDL overlay. These data indicate that an overlay of PDL is able to alter the differentiation of neural crest cells even if they are initially plated on an FN substrate.

5 Moreover, they suggest that at least some of the N+G+O clones derived by conversion of founder cells that would have produced G+O clones on FN. However, because of the increased cytotoxicity obtained from the PDL overlay, it was not possible to rule out the

10 possibility that many of the cells that would have produced G+O clones simply died. To address this issue, the PDL overlay was performed on a parallel set of cultures at day 5 rather than at 48 hrs. Under these conditions, virtually all of the LNGFR⁺ clones

15 survived and differentiated. 60% of these clones contained neurons, whereas 35% contained GAO (Figure 14, panel B). By contrast, greater than 90% of the clones maintained on FN developed to a G+O phenotype. Since little or no clone death was obtained under these

20 conditions, and since a majority of the clones contained neurons following the PDL overlay at day 5, these data suggest that PDL converts presumptive G+O clones into N+G+O clones. However the fact that 35% of the clones became G+O following PDL overlay at days,

25 whereas virtually none did so when the overlay was performed at 48 hrs (Fig. 14, compare G+O, hatched bars, in panels A and B), suggests that some clones might become resistant to the effect of PDL between 48 hrs and days.

EXAMPLE 6Substrate Influences Latent Developmental Potential of Neural Crest Cells

To demonstrate more directly that the substrate can alter the developmental fate of neural crest cells, a serial subcloning experiment was performed. Clones were established on FN, and after 5 days the progeny of each clone were subdivided and cloned onto both FN and PDL/FN substrates. Following 10 days of culture in standard medium, the clones were shifted to SCD medium for an additional week to ten days and then fixed, stained and scored for the presence of neurons and Schwann cells. Five of seven primary clones founded on FN gave rise to secondary clones containing neurons when replated onto a PDL/FN substrate at days (Table II). On average, $57 \pm 17\%$ of the secondary clones contained neurons. By contrast, none of the sister secondary clones replated onto FN contained neurons (Table II). These data confirm that the PDL/FN substrate is able to alter the fate of neural crest cell clones initially grown on FN. They also reveal that the "neurogenic potential" of neural crest cells is retained, at least for a period of time, on FN even though overt neuronal differentiation is not observed. This suggested that FN is non-permissive for overt neuronal differentiation under these culture conditions. In support of this idea, when primary clones established on PDL/FN were replated onto FN, none of the secondary clones contained neurons, whereas 100% (5/5) of the primary clones gave rise to neuron-containing secondary clones when replated onto PDL/FN (Table II). Moreover, on average $93 \pm 7\%$ of the secondary clones derived from each primary clone contained neurons on PDL/ FN, indicating that most or

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all of the clonogenic secondary crest cells retained neurogenic potential under these conditions.

While this experiment indicated that at least some neural crest clones retain neurogenic potential on FM,
5 not all clones exhibited this capacity. This could indicate a heterogeneity in the clonogenic founder cells that grow on FN, or it could indicate a progressive loss of neurogenic potential with time in culture on FM. To address this issue, a second
10 experiment was performed in which primary clones were replated at day 8 rather than at day 5. In this case, a more dramatic difference was observed between primary clones established on FM versus on PDL/FN. Only 1/6 primary FM clones replated at day 8 gave rise to any
15 secondary clones containing neurons on PDL/FN, and in this one case only 17% of the secondary clones contained neurons (Table II). By contrast, 6/6 primary PDL/FN clones gave rise to neuron-containing secondary clones when replated on PDL/FN at day 8, and 52+7% of
20 these secondary clones contained neurons (Table II). These data suggest that neurogenic potential is gradually lost by neural crest cells cultured on FM, but retained to a much greater extent by the same cells grown on PDL/FN. Thus the composition of the substrate
25 influences not only the overt differentiation of the neural crest cells, but also their ability to maintain a latent developmental potential over multiple cell generations.

EXAMPLE 7

Identification of
Neural Crest Stem Cells by Transplantation

5 Neural crest stem cells are identified by two general criteria: by their antigenic phenotype, and by their functional properties. These functional properties may be assessed in culture (*in vitro*), as described above, or they may be assessed in an animal (*in vivo*). The above examples described how the self-renewal and
10 differentiation of neural crest stem cells can be assayed *in vitro*, using clonal cell cultures. However, these properties may also be determined by transplanting neural crest cells into a suitable animal host. Such an assay requires a means of delivering the
15 cells and of identifying the transplanted cells and their progeny so as to distinguish them from cells of the host animal. Using standard techniques, it is possible to deliver neural crest cells to a developing mammalian or avian embryo or to any tissue or
20 compartment of the adult animal (e.g., brain, peritoneal cavity, etc.).

For example, neural crest cell cultures are prepared as described earlier. After a suitable period in primary or secondary culture, neural crest cells are identified
25 by live cell-labeling with antibodies to LNGFR, and removed from the plate using trypsin and a cloning cylinder, as described in previous examples. The cells are diluted into serum-containing medium to inhibit the trypsin, centrifuged and resuspended to a concentration
30 of 10^6 - 10^7 cells per milliliter. The cells are maintained in a viable state prior to injection by applying them in small drops (ca. 10 μ l each) to a 35 mm petri dish, and evaporation is prevented by overlaying the droplets with light mineral oil. The

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cells are kept cold by keeping the petri dishes on ice. For injections into mouse embryos, pregnant mothers at embryonic day 8.5 - 9.0 are anaesthetized and their uterus exposed by an incision into the abdomen. Neural crest cells are drawn into a sharpened glass micropipette (with a sealed tip and hole in the side to prevent clogging during penetration of tissues) by gentle suction. The pipette is inserted into the lower third of the deciduum and a volume of approximately 0.5 µl is expelled containing approximately 1000 cells. The micropipette is withdrawn and the incision is sutured shut. After an additional 3-4 days, the mother is sacrificed, and individual embryos are removed, fixed and analyzed for the presence and phenotype of cells derived from the injected neural crest cells.

To identify the progeny of the injected cells, it is necessary to have a means of distinguishing them from surrounding cells of the host embryo. This may be done as follows: rat neural crest cells are injected into a mouse embryo (following suitable immunosuppression of the mother or using a genetically immunodeficient strain such as the SCID strain of mice), the injected cells are identified by endogenous markers such as Thyl or major histocompatibility complex (MHC) antigens using monoclonal antibodies specific for the rat Thyl or MHC antigens. Alternatively, an exogenous genetic marker is introduced into the cells prior to their transplantation as a means of providing a marker on or in the injected cells. This is as follows: neural crest cells in culture are incubated with a suspension of replication-defective, helper-free retrovirus particles harboring the lacZ gene, at a titer of 10^5 - 10^6 pfu/ml in the presence of 8 µl/ml polybrene for four hours. The cells are then washed several times with fresh medium and prepared for injection as described above. The harvested embryos are then

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assayed for expression of β -galactosidase by whole mount staining according to standard procedures. The blue cells (indicating expression of the lacZ gene) will correspond to the progeny of the injected neural
5 crest cells. This procedure can be applied to any tissue or any stage of development in any animal suitable for transplantation studies. Following whole-mount staining, embryos bearing positive cells are embedded in freezing medium and sectioned at 10-20 μm
10 on a cryostat. Sections containing blue cells are selected, and then counterstained for markers of mature neurons and glia using specific antibodies, according to standard techniques, and immunoperoxidase or alkalinephosphatase histochemistry. The identification
15 of lacZ+ (blue) cells expressing neuronal or glial markers indicates that the progeny of the injected neural crest cells have differentiated appropriately. Thus, this technique provides a means of identifying mammalian neural crest stem cells through
20 transplantation studies to reveal the function of said stem cells.

EXAMPLE 8

Genetic-Engineering of
Neural Crest Stem Cells (NCSCs)

25 A. Retroviral infection of NCSCs

In this method, NCSCs are infected with a replication-incompetent, recombinant retrovirus harboring the foreign gene of interest. This foreign gene is under the control of the long terminal repeats (LTRs) of the
30 retrovirus, in this case a Moloney Murine Leukemia Virus (MoMuLv) (Cepko et al. (1984) Cell 37:1053-1062). Alternatively, the foreign gene is under the control of a distinct promoter-enhancer contained within the

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recombinant portion of the virus (*i.e.*, CMV or RSV LTR). In this particular example, the *E. coli* β -galactosidase gene was used, because it provides a blue histochemical reaction product that can easily be used 5 to identify the genetically-engineered cells, and thereby determine the transformation efficiency.

Rat NCSC cultures were established as described above. Twenty-four hours after replating, the cells were exposed to a suspension of β -galactosidase-containing 10 retrovirus (Turner et al. (1987) Nature 328:131-136) with a titer of approximately 10^5 - 10^6 pfu/ml in the presence of 8 μ g/ml polybrene. Following a 3 hr exposure to the viral suspension, the cultures were rinsed and transferred into standard medium. After 15 three days of growth in this medium, the transformed cells were visualized using the X-gal histochemical reaction (Sanes et al. (1986) EMBO J. 5:3133-3142) Fig. 15, Panel A shows the NCSC culture three days after infection with the lacZ containing retrovirus, after 20 fixation and staining using the X-gal reaction. β -galactosidase-expressing cells are indicated by the solid arrows. Non-expressing cells in the same microscopic field are visualized by phase contrast microscopy (B), and are indicated by open arrows. The 25 blue, β -galactosidase⁺ cells represented approximately 5-10% of the total cells in the culture as visualized by phase-contrast microscopy (Fig. 15, Panel B).

B. Calcium-Phosphate-Mediated Transfection of NCSCs

In this method, NCSCs are transfected with an 30 expression plasmid using the calcium phosphate method (Wigler et al. (1979) Proc. Natl. Acad. Sci. USA 76:1373-1376). As in the previous example, the β -galactosidase gene was used to facilitate visualization of the transfected cells.

In this case, the vector pRSVlacZ was used, in which the β -galactosidase gene (lacZ) is under the control of the Rous Sarcoma Virus (RSV) LTR, and the SV40 intron and poly A-addition site are provided at the 3' end of
5 the gene (Johnson et al. (1992) Proc. Natl. Acad. Sci. USA 89:3596-3600).

NCSCs were established in 35 mm tissue culture dishes. 24 hr after plating, a calcium phosphate precipitate containing approximately 20 $\mu\text{g}/\text{ml}$ of pRSVlacZ was prepared. 123 μl of this precipitate was added to each dish, and incubated at room temperature for 20 minutes. Two ml of standard medium containing 30 μM chloroquine was then added to each dish and incubation was continued overnight at 37°C. The next day, the medium 10 was replaced and incubation continued for a further two days. The cultures were then fixed and assayed for β -galactosidase expression by the standard X-gal reaction. Approximately 10% of the NCSCs expressed the lacZ reaction product.
15

20 C. Immortalization of NCSCs

NCSC cultures are established as described above. The cultures are exposed, in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene, to a suspension of retrovirus harboring an oncogene preferably selected from the immortalizing 25 oncogenes identified herein. These retroviruses contain, in addition to the oncogene sequences, a gene encoding a selectable marker, such as *hisD*, driven by the SV40 early promoter-enhancer (Stockschlaeder, M.A.R. et al. (1991) Human Gene Therapy 2:33). Cells 30 which have taken up the *hisD* gene are selected for by growth in the presence of L-histidinol at a concentration of 4 mM. Alternatively, selection can be based upon growth in the presence of neomycin (500 $\mu\text{g}/\text{ml}$). NCSCs are infected with the above 35 retroviruses which are concentrated to a titer of

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greater than 10^6 pfu/ml by centrifugation. The virus is applied to the cells in two sequential incubations of 4-8 hours each in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene.

Following infection, the cells are grown in the
5 presence of 4 mM L-histinol or 500 $\mu\text{g}/\text{ml}$ neomycin
(G418) for 5-10 days. Cells which survive the
selection process are screened for expression of LNGFR
by live-cell labeling using the monoclonal antibody 192
Ig as described above. Colonies containing a
10 homogeneous population of LNGFR+ cells are cloned using
a cloning cylinder and mild trypsinization, and
transferred into duplicate FN/pDL-coated 96-well
plates. After a short period of growth, one of the
plates is directly frozen (Ramirez-Solis, R. et al.
15 (1992) Meth. Enzymol., in press). The cells in the
other plate are replated onto several replicate 96-well
plates, one of which is maintained for carrying the
lines. The cells on the other plates are fixed and
analyzed for the expression of antigenic markers.
20 Successful immortalization is indicated by (1) the
cells homogeneously maintain an antigenic phenotype
characterized by LNGFR+, nestin+, lin- (where "lin"
refers to lineage markers characteristic of
differentiated neuronal or glial crest derivatives,
25 including neurofilament, peripherin, hi PSA-NCAM, GFAP,
O4 and P₀); and (2) the cell population is
phenotypically stable over several weeks of passage (as
defined by lack of differentiation to morphologically-
and antigenically-recognizable neurons and/or glia).
30 The ability of the lines to differentiate is tested by
transferring them to conditions that promote
differentiation (omission of CEE in the case of neurons
and addition of serum and 5 μM forskolin for Schwann
cells). Maintenance of the ability to differentiate is
35 a desirable, although not necessary, property of the
constitutively-immortalized cells.

EXAMPLE 9Generation of Monoclonal Antibody to Mouse LNGFR

Mouse monoclonal antibodies specific to LNGFR from primates (Loy et al. (1990), J. Neurosci. Res. 27:657-664) and rat (Chandler et al. (1984) J. Biol. Chem. 259:6882-6889) have been produced. No monoclonal antibodies to mouse LNGFR have been described. We have produced rat monoclonal antibodies to mouse LNGFR. These antibodies recognize epitopes present on the surface of living cells such as Schwann cells, making them suitable for use in immunologic isolation of multipotent neural stem cells (such as neural crest stem cells) and their differentiated derivatives (as well as neural progenitor cells from the CNS) from murine species. The isolation of such cells from mice is particularly desirable; as that species is the experimental organism of choice for genetic and immunological studies or human disease.

To generate monoclonal antibodies to mouse LNGFR, a genomic DNA fragment encoding the extracellular domain (ligand binding domain) of that protein was expressed in *E. coli*, as a fusion protein with glutathione-S-transferase (Lassar et al. (1989) Cell 58:823-831). Briefly, a probe for the extracellular domain based on either of the known DNA sequences for rat and human LNGFR is used to screen a mouse genomic library. A cloned insert from a positively hybridizing clone is excised and recombined with DNA encoding glutathione with appropriate expression regulation sequences and transfected into *E. coli*. The fusion protein was affinity-purified on a glutathione-Sepharose column, and injected into rats. Sera obtained from tail bleeds of the rats were screened by surface-labeling of live Schwann cells isolated from mouse sciatic nerve by

standard procedures (Brockea et al. (1979) In Vitro 15:773-778. Surface labeling was with labelled goat anti-rat antibody. Following a boost, fusions were carried out between the rat spleen cells and mouse myeloma cells. Supernatants from the resulting hybridoma cultures were screened using the live Schwann cell assay. Positive clones were re-tested on NIH 3T3 fibroblasts, a mouse cell line that does not express LNGFR, and were found to be negative. The use of this live cell assay ensures that all antibodies selected are able to recognize LNGFR on the surface of living cells. Moreover the assay is rapid, simple and more efficient than other assays such as ELISA, which require large quantities of purified antigen.

Approximately 17 independent positive hybridoma lines were identified and subcloned. An example of the results obtained with the supernatant from one such line is shown in Figure 16. A culture of mouse sciatic nerve Schwann cells was labeled with one of the rat anti-mouse LNGFR monoclonal antibodies and counterstained with DAPI to reveal the nuclei of 611 cells. The left panel (A) shows that most of the cells are labeled on their surface with the anti-LNGFR antibody (red staining; solid arrows), the right panel (B) reveals all the cell nuclei on the plate, and shows a few cells not labeled by the anti-LNGFR antibody (blue staining; open arrows; compare to left panel). These unlabeled cells most likely represent contaminating fibroblasts which are known not to express LNGFR. These cells provide an internal control which demonstrates the specificity of the labeling obtained with the anti-LNGFR antibody.

WHAT IS CLAIMED IS:

1. An isolated cellular composition comprising at least one mammalian multipotent neural stem cell capable of self-renewal in a feeder cell-independent culture medium and capable of differentiation to at least neuronal or glial progenitor cells.
5
2. A cellular composition according to Claim 1, wherein said stem cell is capable of differentiating to at least peripheral nervous system neuronal or glial progenitor cells and is further characterized by the presence of a low-affinity nerve growth factor receptor⁺.
10
3. A cellular composition according to Claim 2, wherein said stem cell is further characterized by being nestin⁺.
15
4. A cellular composition according to Claim 3, wherein said cells are further characterized as lacking one or more markers characteristic of mature neuronal or glial cells of the peripheral nervous system.
- 20 5. The cellular composition of Claim 4 wherein said markers are selected from the group consisting of sulfatide, glial fibrillary acidic protein, peripherin and a neurofilament.
- 25 6. An isolated cellular composition comprising a mammalian peripheral nervous system glial progenitor cell.
7. A method for obtaining a cellular composition comprising mammalian neural crest stem cells, said method comprising:

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a) contacting an embryonic neural tube with a first culture medium and a first substrate to produce a suspension comprising a population of neural crest cells that have emigrated from said embryonic neural
5 tube and

b) contacting at least a portion of said suspension comprising one or more cells of said population with a second culture medium and a second substrate which permit self-regeneration and
10 differentiation of one or more neural crest stem cells in said population.

8. A method according to Claim 7 further comprising the step:

c) identifying at least one stem cell by its ability to self-renew and differentiate in feeder cell-independent culture.
15

9. The method of Claim 8 further comprising prior to or after step b) the step of identifying at least one neural crest stem cell which is characterized by the presence of low-affinity nerve growth factor receptor
20 on the surface of said neural crest stem cell.

10. The method of Claim 7, wherein said differentiation of said stem cells is to at least peripheral nervous system neuronal or glial progenitor
25 cells.

11. The method of Claim 7, wherein said first and second substrates comprise fibronectin.

12. The method of Claim 7, wherein said first and second substrates comprise poly-D-lysine in combination
30 with fibronectin.

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13. The method of Claim 7, wherein said second culture medium further comprises a factor permissive for peripheral nervous system neuronal cell differentiation.

5 14. The method of Claim 13, wherein said factor permissive for peripheral nervous system neuronal cell differentiation is a substrate comprising poly-D-lysine in combination with fibronectin.

10 15. The method of Claim 7, wherein said second culture medium further comprises a factor permissive for peripheral nervous system glial cell differentiation.

16. The method of Claim 15, wherein said factor permissive for peripheral nervous system glial cell differentiation is a substrate comprising fibronectin.

15 17. The method of Claim 15, wherein said factor permissive for peripheral nervous system glial cell differentiation is forskolin.

20 18. An isolated cellular composition comprising a mammalian peripheral nervous system neuronal progenitor cell made according to the method of Claim 13.

19. An isolated cellular composition comprising a mammalian peripheral nervous system neuronal cell made according to the method of Claim 13.

25 20. An isolated cellular composition according to Claim 19, wherein said neuronal cells are characterized by being nestin⁺, low-affinity nerve growth factor receptor⁺, peripherin⁺, and neurofilament⁺.

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21. An isolated cellular composition comprising mammalian peripheral nervous system glial progenitor cells made according to the method of Claim 15.

22. An isolated cellular composition comprising
5 mammalian peripheral nervous system glial cells made according to the method in Claim 15.

23. An isolated cellular composition according to
Claim 22, wherein said glial cells are characterized as
being low-affinity nerve growth factor receptor⁺,
10 nestin⁺, glial fibrillary acidic protein⁺, and
sulfatide⁺.

24. A method for obtaining a cellular composition from
a mammalian tissue comprising one or more cells having
at least one property characteristic of a glial or
15 neuronal progenitor cell or multipotent stem cell
precursor of said progenitor cells, said method
comprising:

a) preparing a suspension comprising a population of cells from said mammalian tissue,

20 b) contacting said cell suspension with a first culture medium and a first substrate which permit self-renewal of one or more glial or neuronal progenitor cells or multipotent stem cell precursors, and

c) identifying said glial or neuronal progenitor
25 cells or multipotent stem cell precursors, if present,
by their ability to self-renew and differentiate in
feeder-cell independent culture.

25. A method according to Claim 24 further comprising
the step:

30 c) contacting said glial or neuronal progenitor cells or multipotent stem cell precursors of said progenitor cells with a second culture medium and a second substrate which permit differentiation of one or

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more of said glial or neuronal progenitor cells or multipotent stem cell precursors.

26. A method according to Claim 25 further comprising prior to step b):

5 e) isolating one or more cells expressing a neural cell-specific surface marker.

27. A method according to Claim 26 wherein said neural cell-specific surface marker comprises the low affinity nerve growth factor receptor.

10 28. A method according to Claim 24 wherein said cells have at least one property characteristic of a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof.

15 29. An isolated cellular composition comprising a glial or neuronal progenitor cell or multipotent stem cell precursor thereof made according to the method of Claim 24.

20 30. An isolated cellular composition comprising a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof made according to the method of Claim 28.

25 31. A method for obtaining a cellular composition from a mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor of said progenitor cells, said method comprising:

a) preparing a suspension comprising a population of cells from said mammalian tissue and

30 b) transplanting at least of cell of said population into a suitable host animal and

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c) identifying a glial or neuronal progenitor cell or multipotent stem cell precursor, if present, by the ability of said cell to self-renew and differentiate into at least the glial and neuronal
5 lineages *in vivo*.

32. A method according to Claim 31 further comprising prior to step b):

d) isolating one or more expressing a neural cell-specific surface marker.

10 33. A method according to Claim 32 wherein said neural cell-specific surface marker comprises the low-affinity nerve growth factor receptor.

15 34. A method for obtaining a cellular composition from a mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor of said progenitor cells, said method comprising:

20 a) preparing a suspension comprising cells from said mammalian tissue,

25 b) contacting said suspension with an antibody capable of forming a first complex with a neural cell-specific surface marker on said glial or neuronal progenitor cells or multipotent stem cell precursor, and

c) isolating said first complex, if formed, from said suspension to obtain said cellular composition.

30 35. A method according to Claim 34 wherein said neural cell-specific surface marker comprises the low-affinity nerve growth factor receptor.

36. A method according to Claim 34 wherein said isolating comprises forming a second complex by

contacting said first complex with a reagent comprising a first member of a binding pair wherein the second member of said binding pair comprises said antibody or a conjugate of said antibody.

5 37. A method according to Claim 36 wherein said reagent contains a fluorescent label and said isolating further comprises separating said second complex, if formed, by fluorescence-activated cell sorting.

10 38. A method according to Claim 34 wherein said one or more cells have at least one property characteristic of peripheral nervous system glial or neuronal progenitor cells or multipotent stem cell precursors of said peripheral nervous system progenitor cells.

15 39. A method comprising introducing one or more cells comprising neuronal or glial progenitor cells or multipotent stem cell precursors thereof into a host animal.

40. A method according to Claim 39 wherein said cells are made according to the method of Claims 24 or 34.

20 41. A method according to Claim 39 wherein said cells comprise peripheral nervous system glial or neuronal progenitor cells or multipotent neural crest stem cell precursors of said peripheral nervous system progenitor cells.

25 42. A method according to Claim 41 wherein said cells are made according to the method of any one of Claims 7, 13, 15 and 29.

43. A method according to Claim 39 or 41 wherein said host animal has a disorder of the nervous system.

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44. A method for immortalizing a cell line having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor thereof comprising transfecting said cell 5 with a vector comprising an immortalizing gene.

45. A method according to Claim 44 wherein said cell line has at least one property characteristic of a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof.

10 46. An immortalized cell line made according to the method of Claim 44 or 45.

47. A cellular composition comprising at least one genetically-engineered multipotent neural stem cell.

15 48. The cellular composition of Claim 47 wherein said stem cell comprises a neural crest stem cell.

49. A cellular composition comprising at least one differentiated progeny of a genetically-engineered multipotent neural stem cell.

50. The cellular composition of Claim 49 wherein said 20 stem cell comprises a neural crest stem cell.

51. A cellular composition according to Claim 50, wherein said progeny of said differentiated progeny comprises a peripheral nervous system neuronal progenitor cell.

25 52. A cellular composition according to Claim 50, wherein said progeny of said differentiated progeny comprises a peripheral nervous system glial progenitor cell.

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53. A method for generating a genetically-engineered multipotent neural stem cell, said method comprising contacting a multipotent neural stem cell with foreign nucleic acid under conditions permissive for the uptake 5 of said foreign nucleic acid into said stem cell.

54. A method according to Claim 53, wherein said conditions permissive for the uptake of said foreign nucleic acid comprise calcium phosphate-mediated transfection.

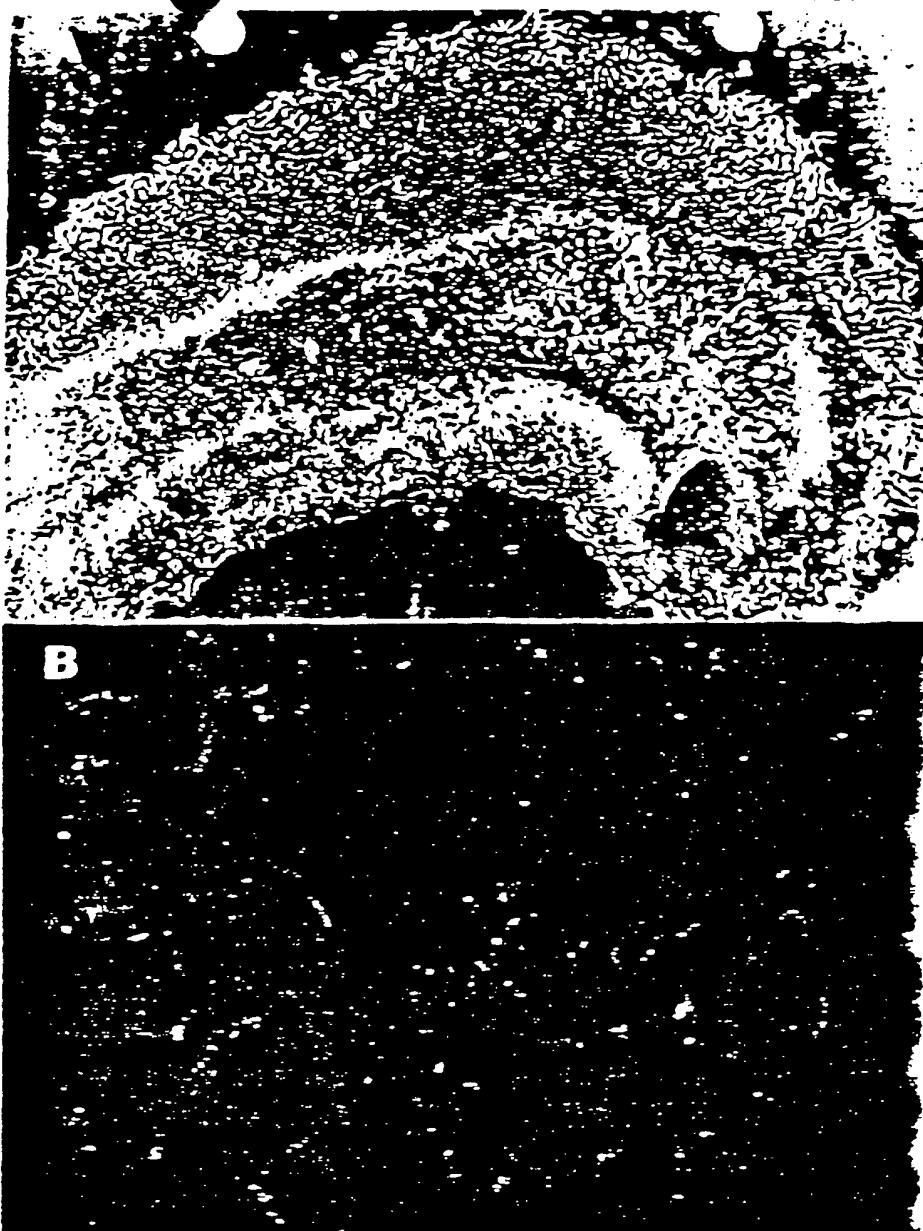
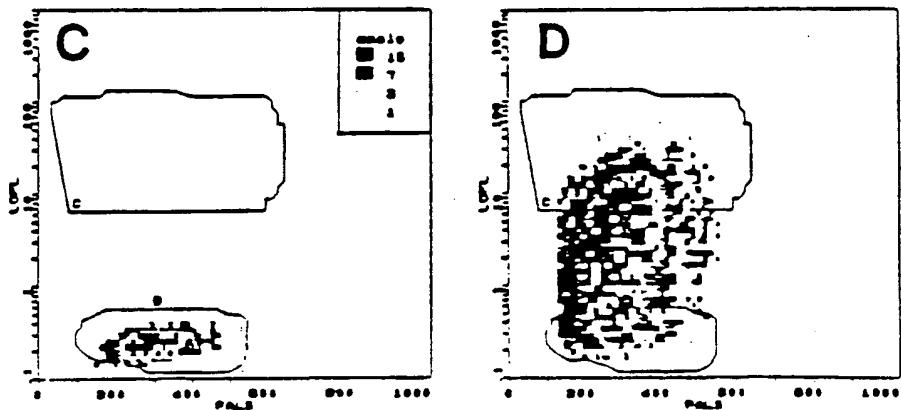
10 55. A method according to Claim 53, wherein said conditions permissive for the uptake of said foreign nucleic acid comprise retroviral infection.

56. A monoclonal antibody capable of binding to mouse low-affinity nerve growth factor receptor.

15 57. A method for detecting the presence of an antibody capable of binding a surface marker characteristic of a neural cell comprising:

immunizing an animal with said surface marker;
contacting a live neural cell displaying said 20 surface marker with antibody-containing sera from said immunized animal or antibody produced by a hybridoma formed from a cell obtained from said immunized animal, and

25 detecting whether antibody specific for said surface marker binds to said neural cell.

**B****Fig 1**

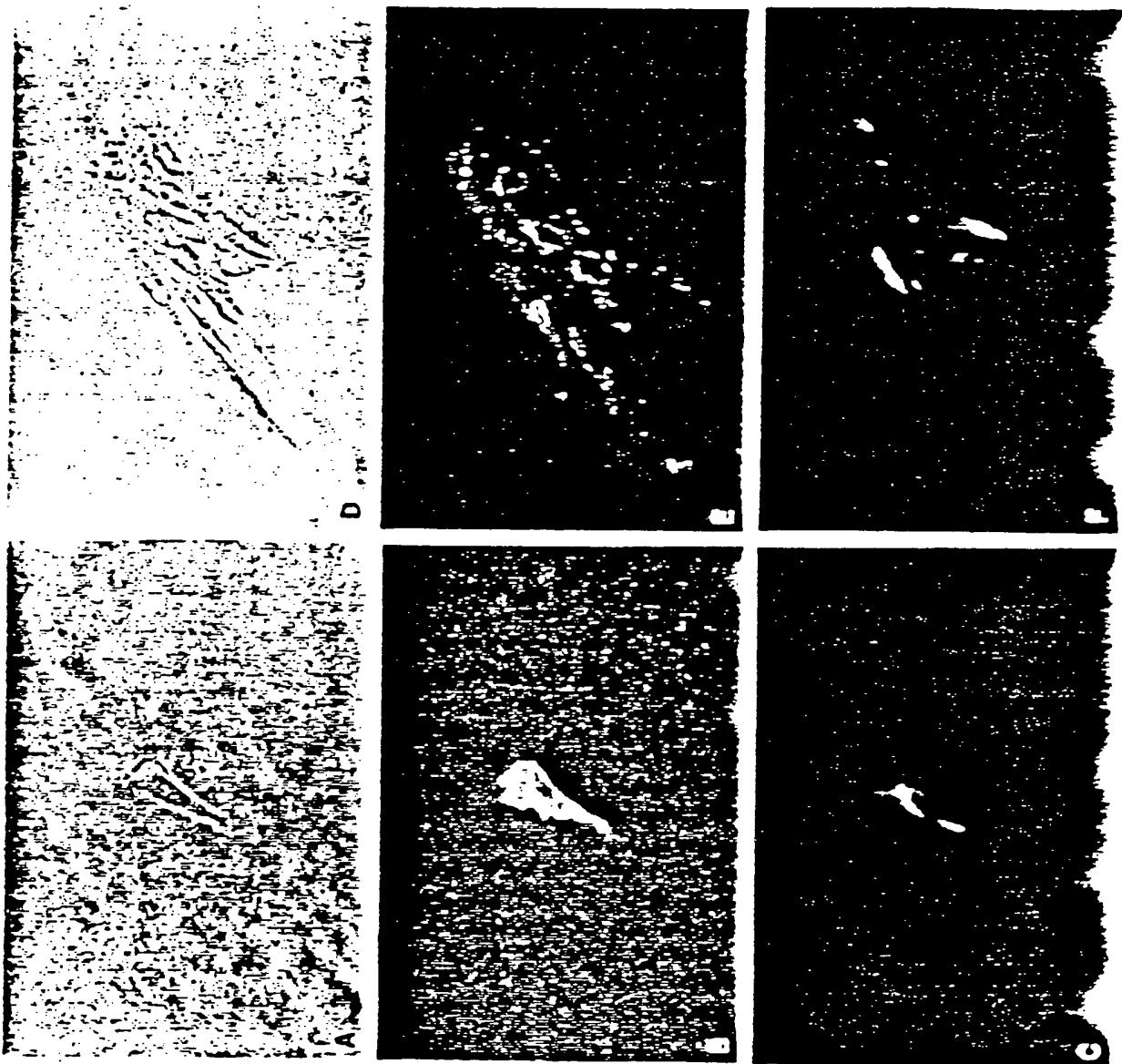


Fig. 2

MULTIPOTENCY OF MAMMALIAN NEURAL CREST CELLS

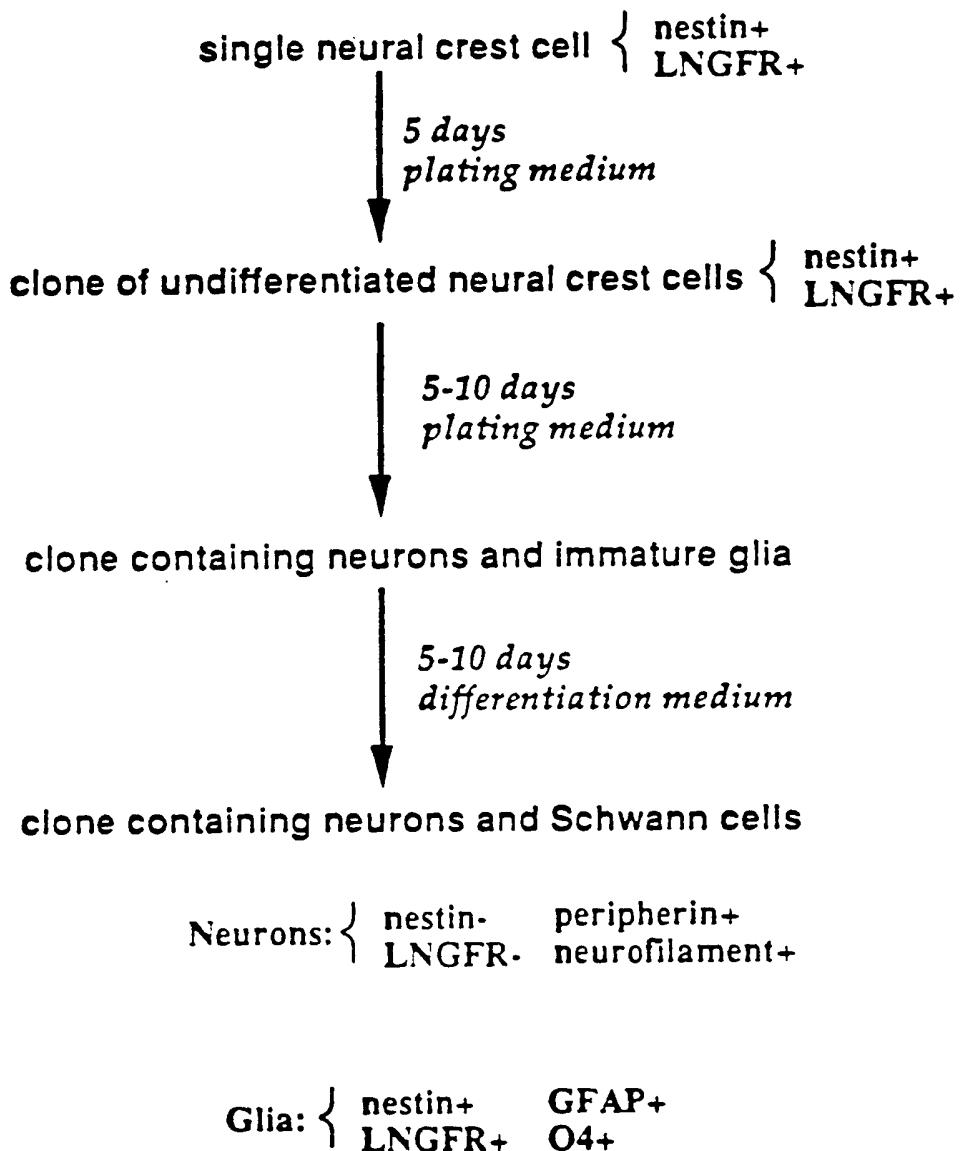


Fig. 3

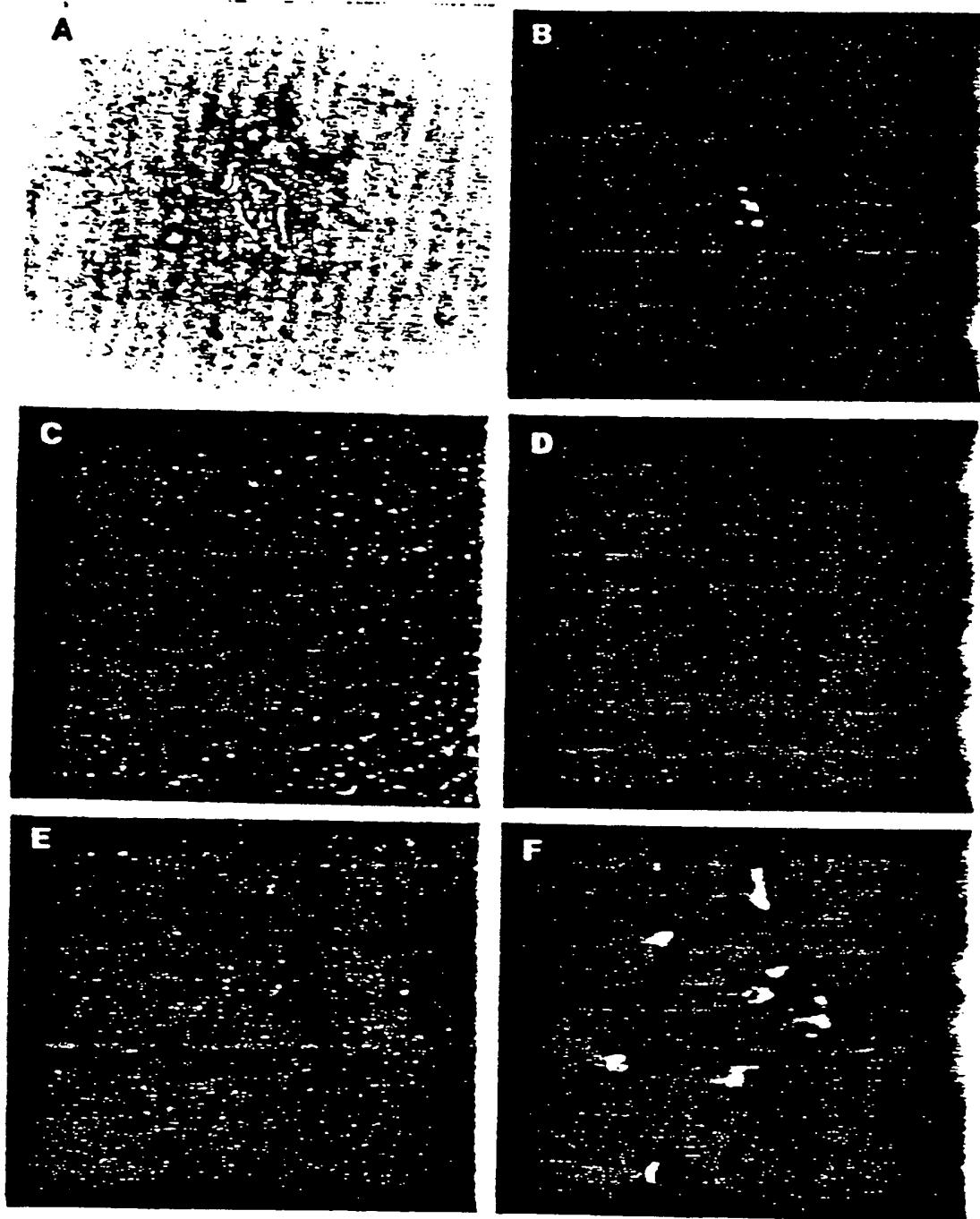


Fig. 4

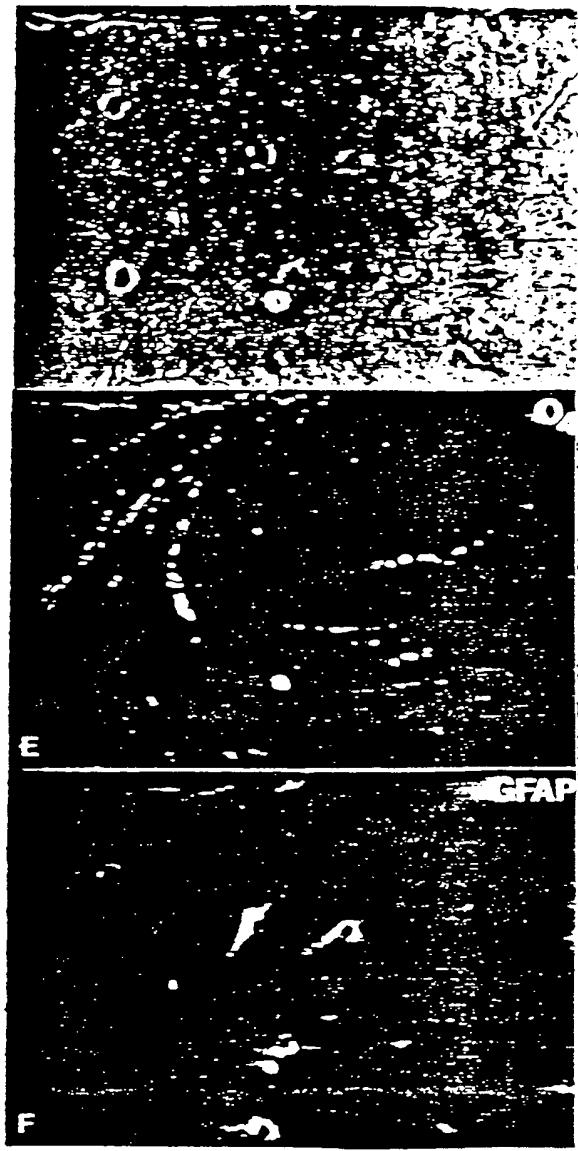
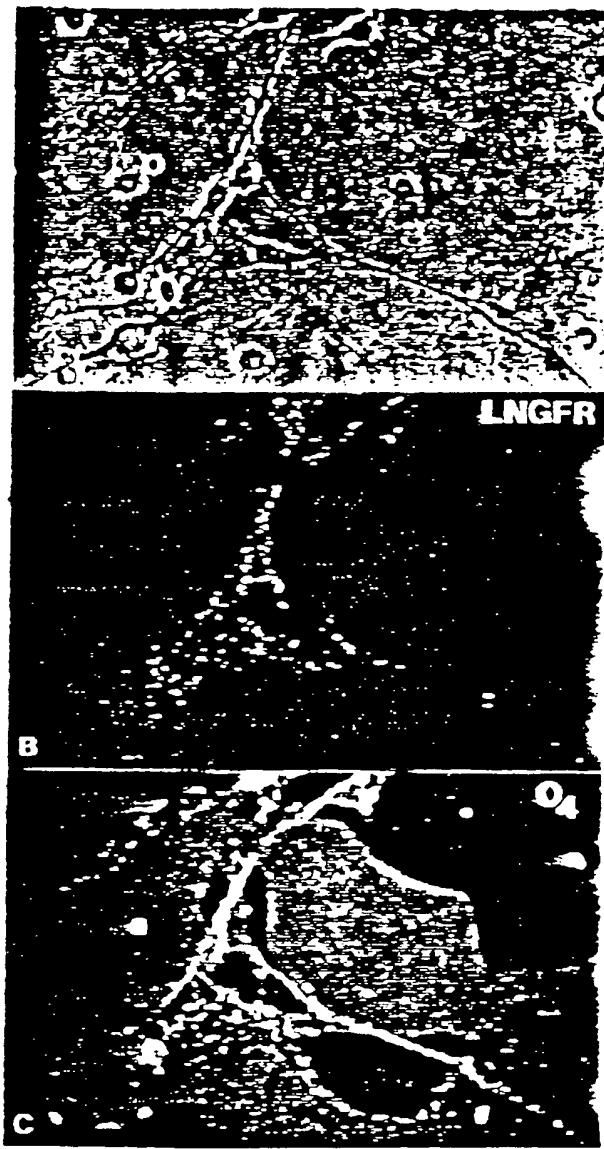


Fig. 5

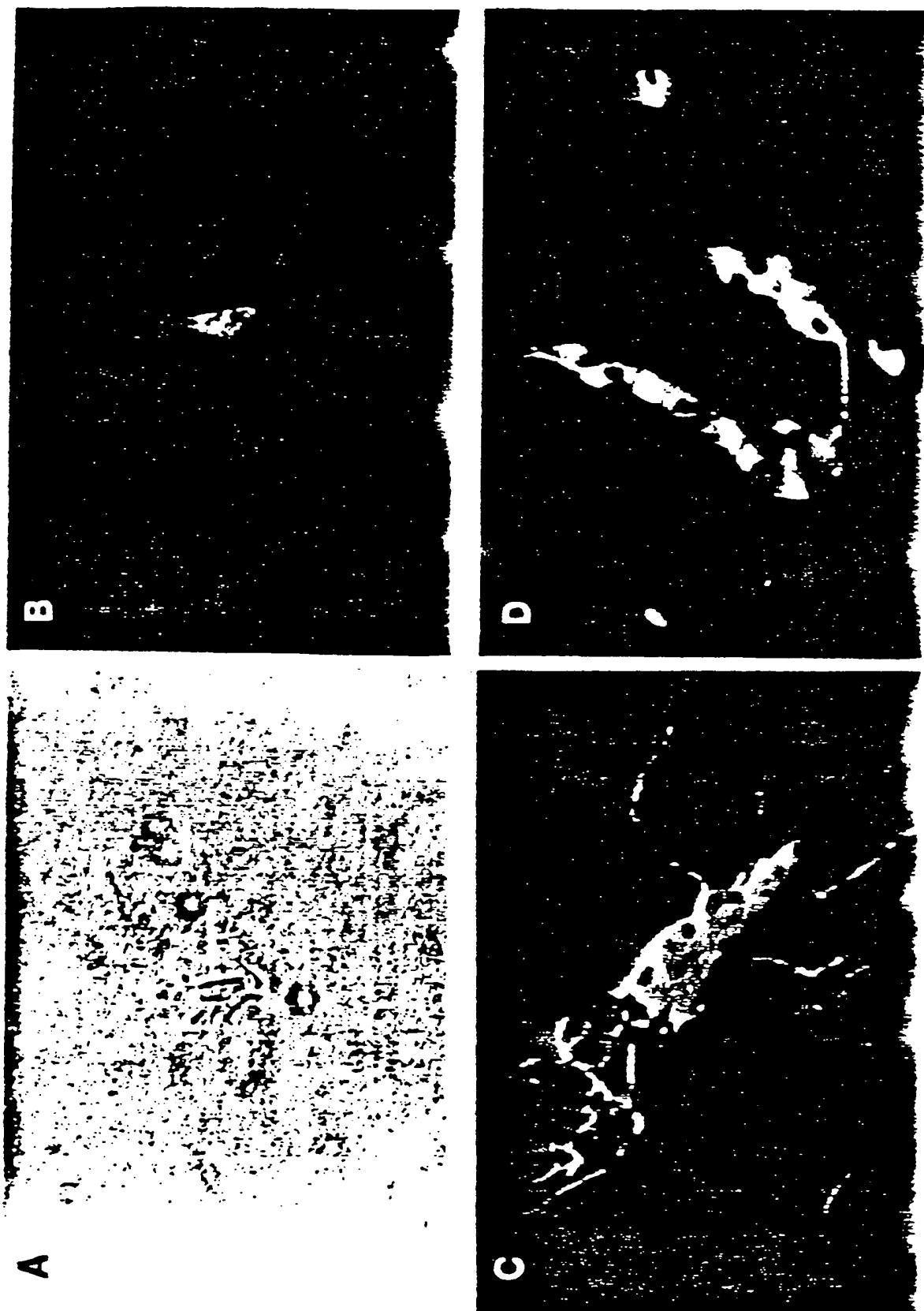


Fig. 6

SELF-RENEWAL OF MAMMALIAN NEURAL CREST CELLS

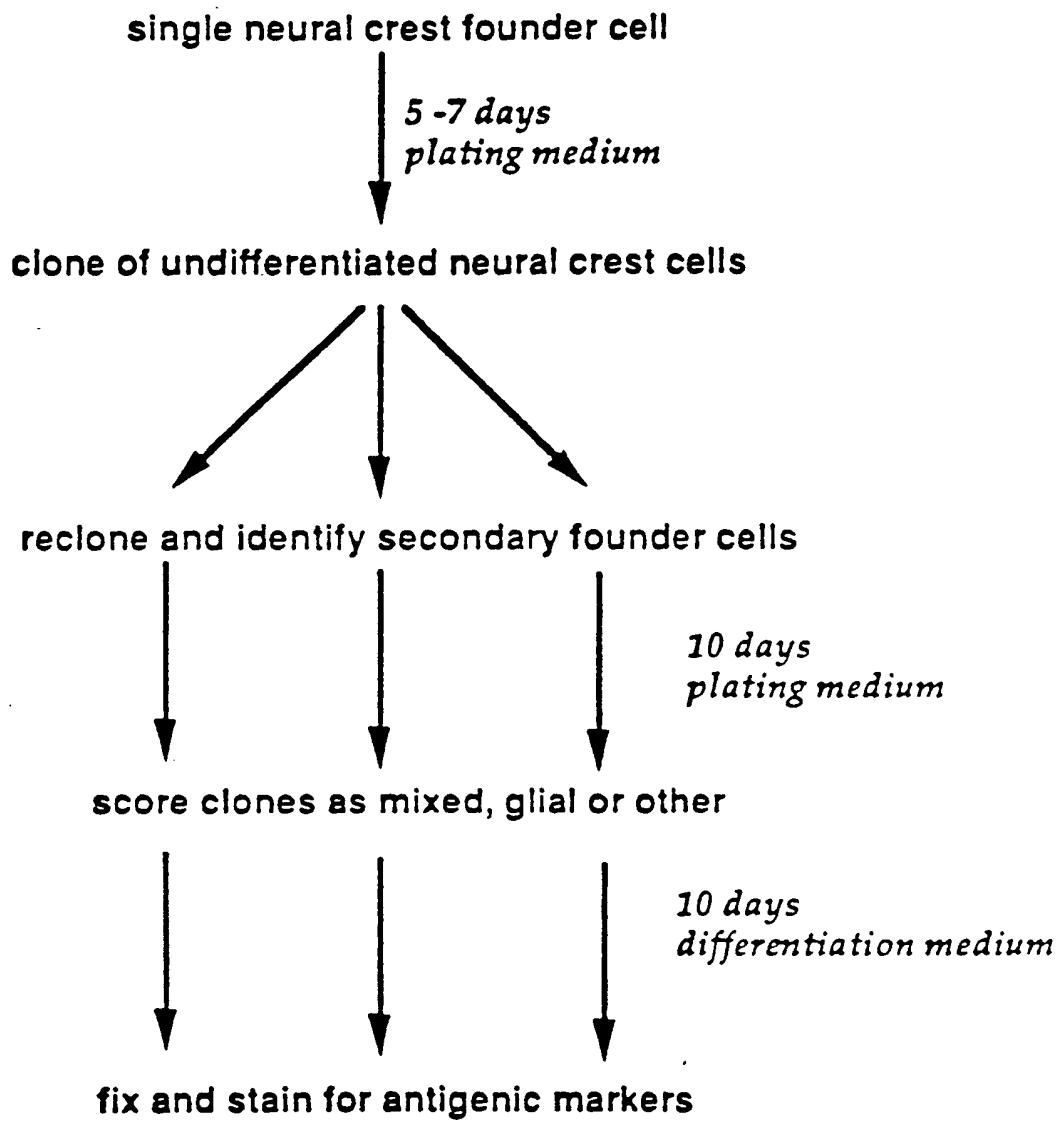
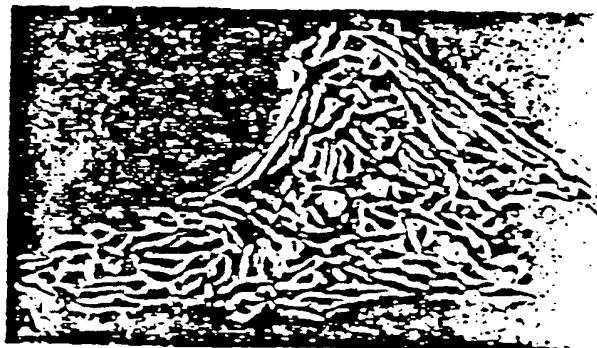
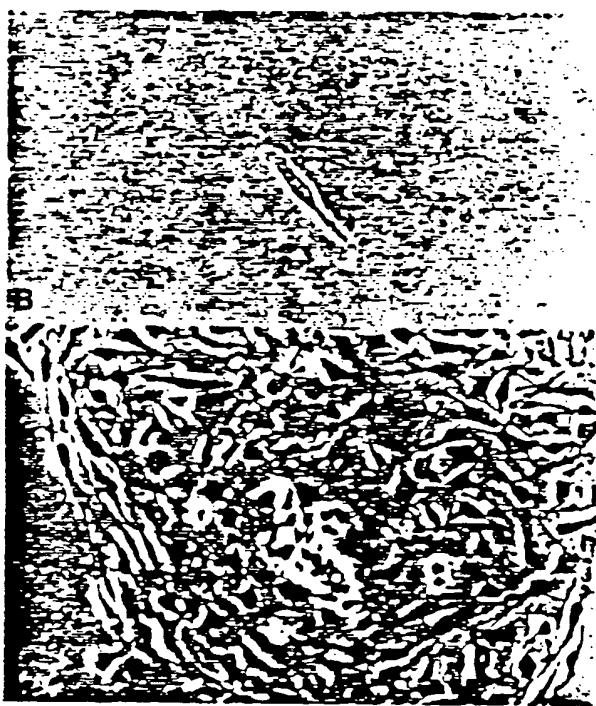


Fig. 7

1 Clone
Day 7



2 Founders



2 Clones
Day 17

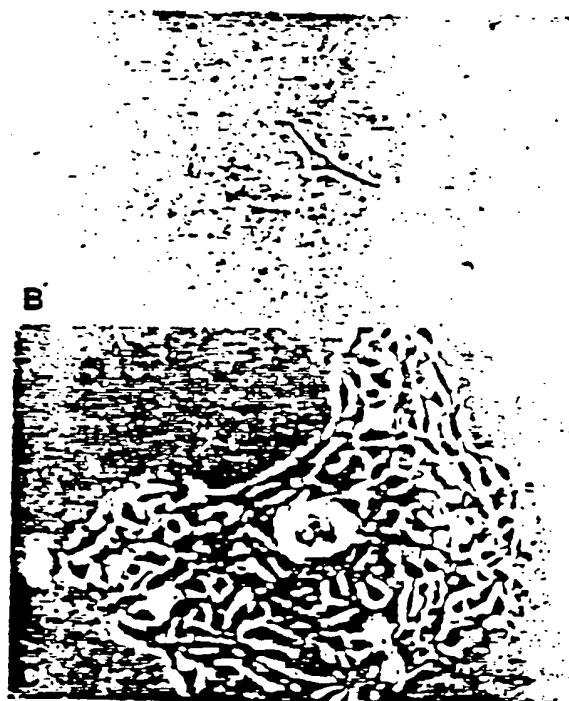
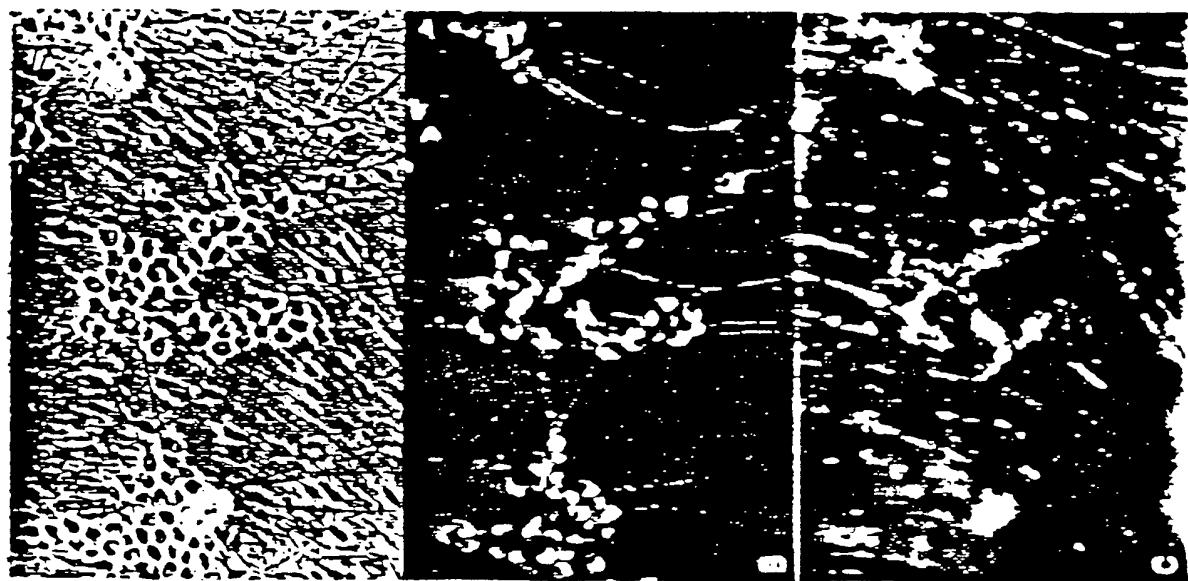


Fig. 8

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NF160

GFAP

Fig. 9

SUBSTRATE EFFECT ON FATE OF MAMMALIAN NEURAL CREST CELLS

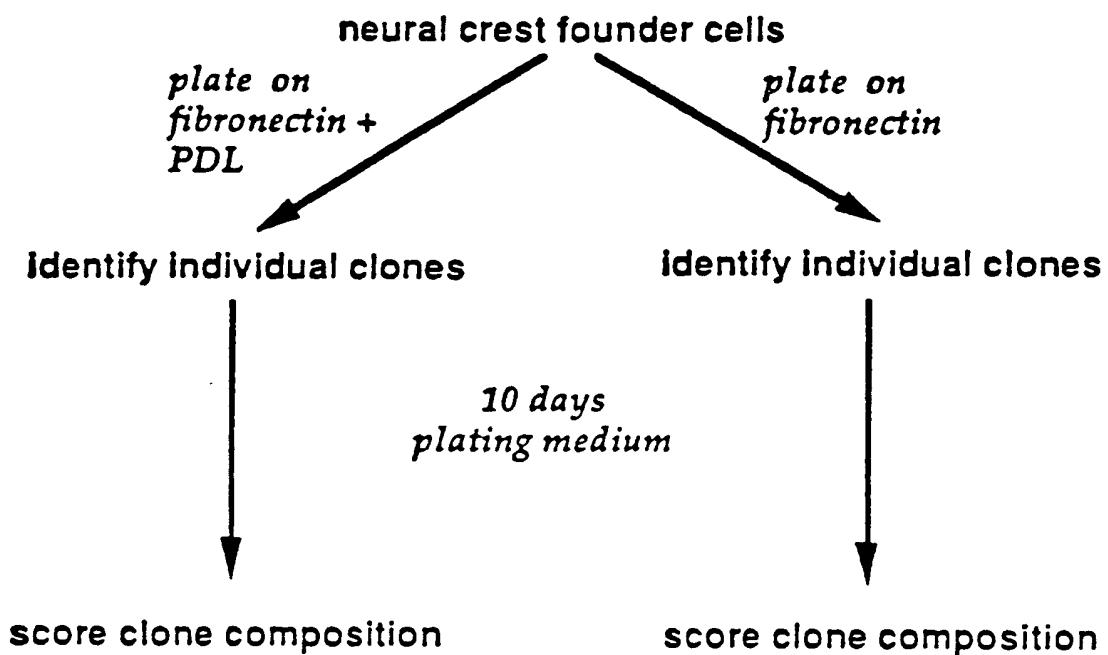


Fig. 10

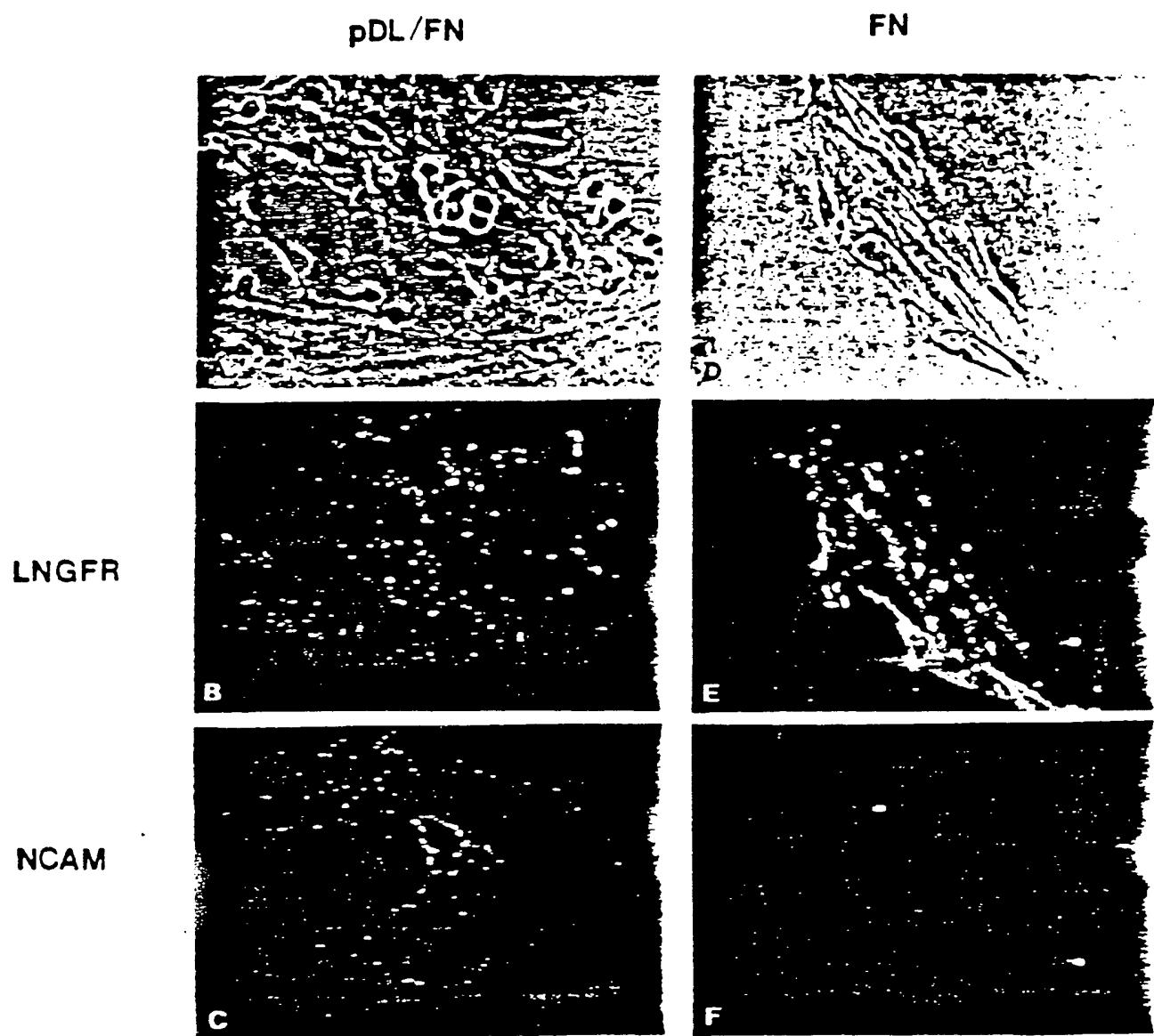


Fig. 11

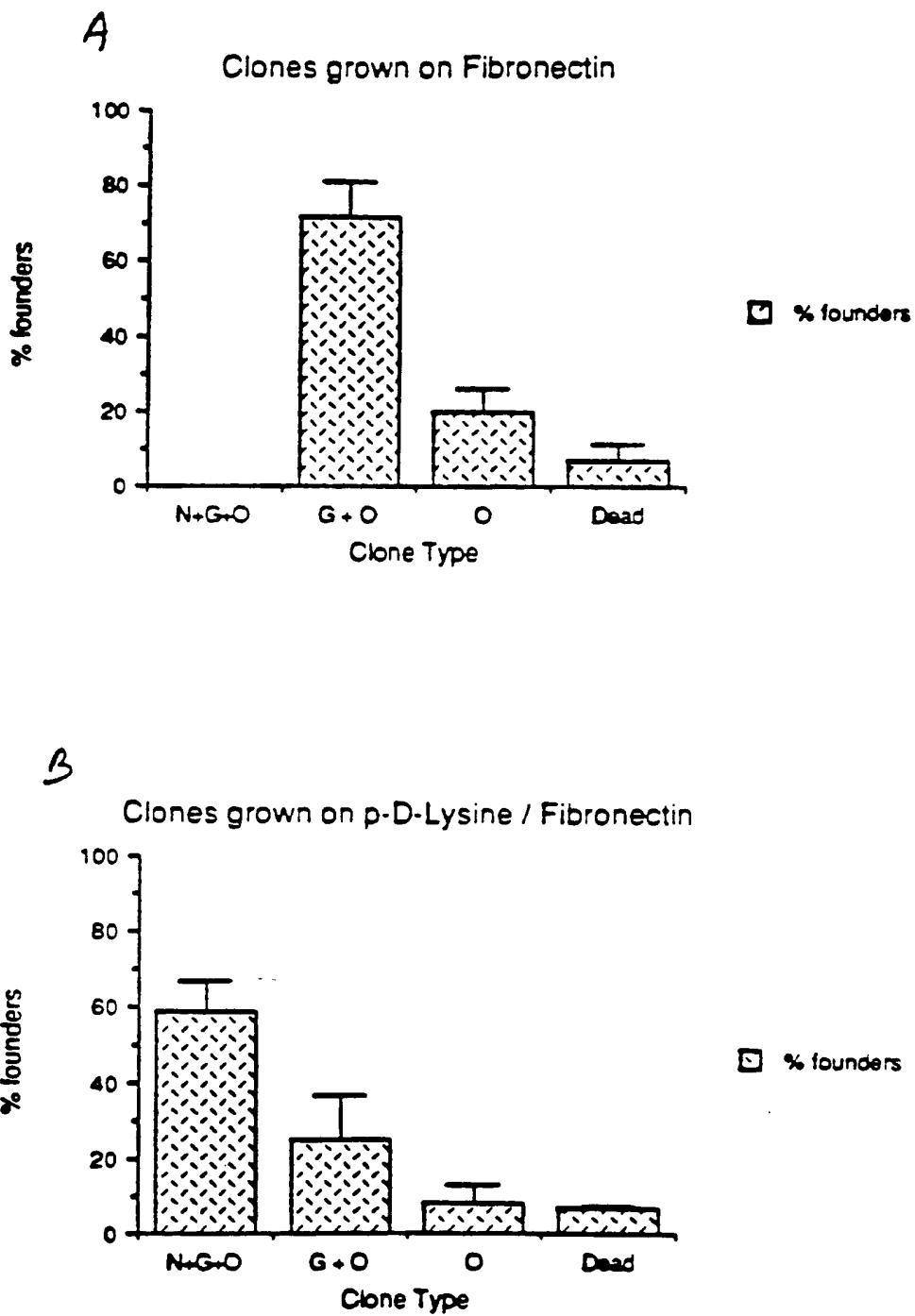


Fig. 12

INSTRUCTIVE EFFECT OF SUBSTRATE ON CREST CELL FATE

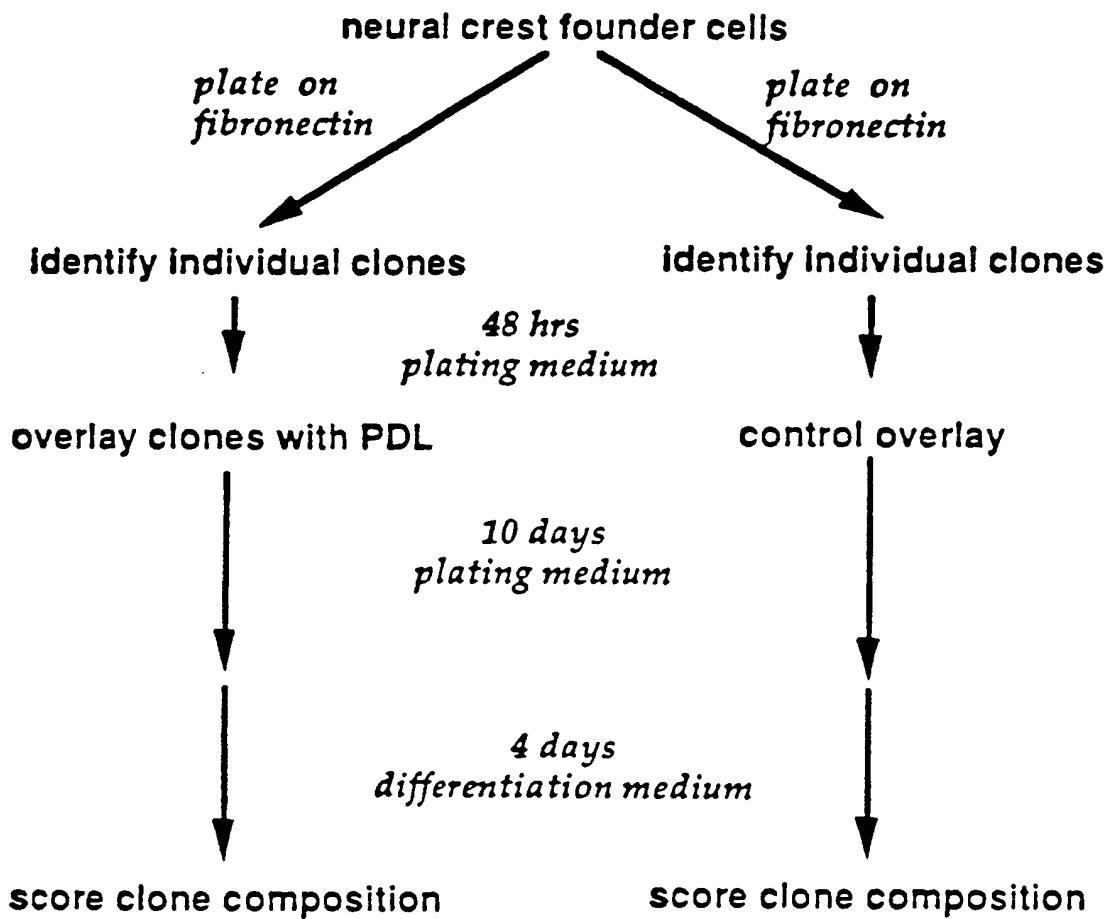
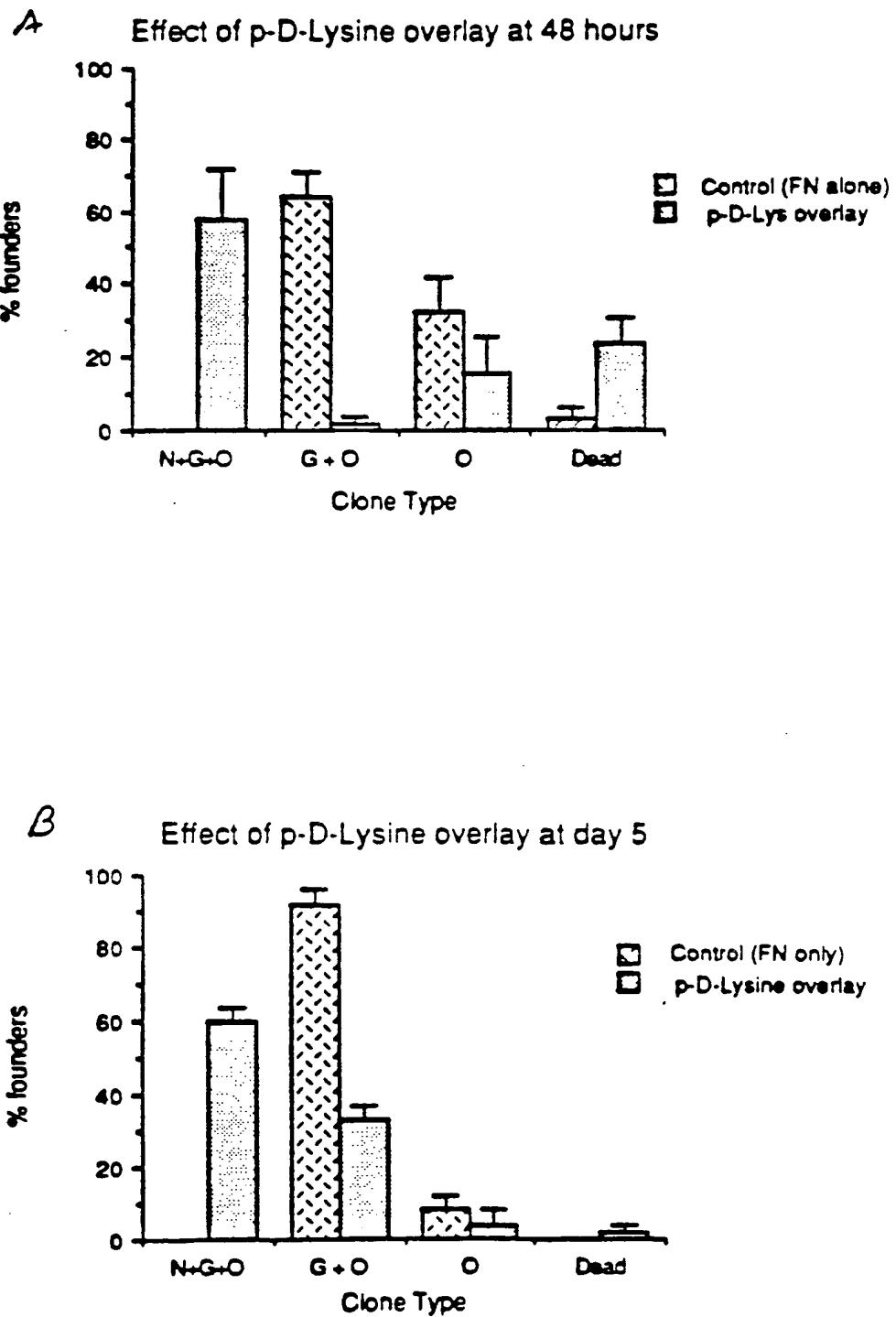


Fig. 13

**Fig. 14**

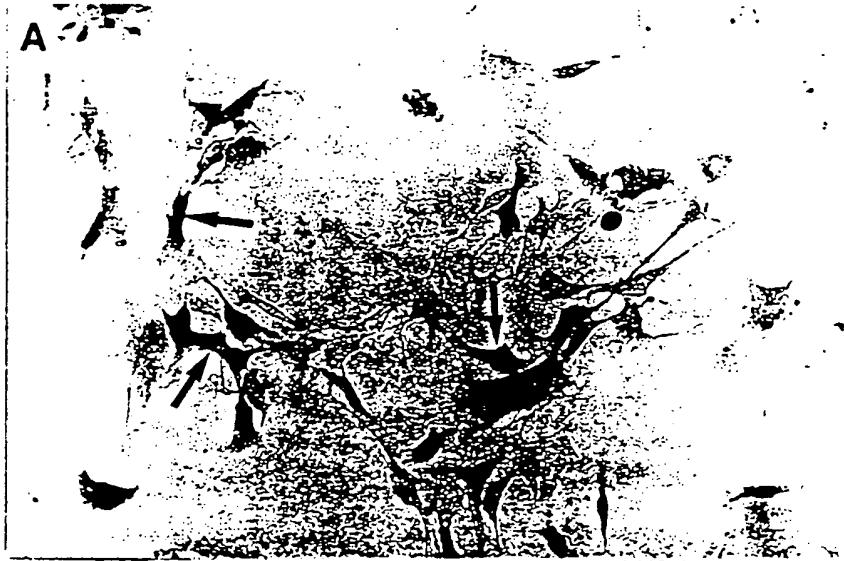
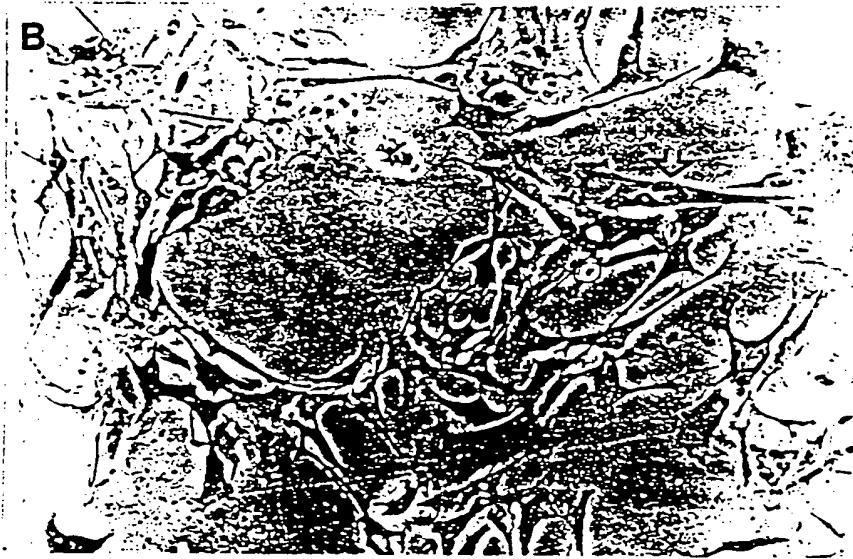
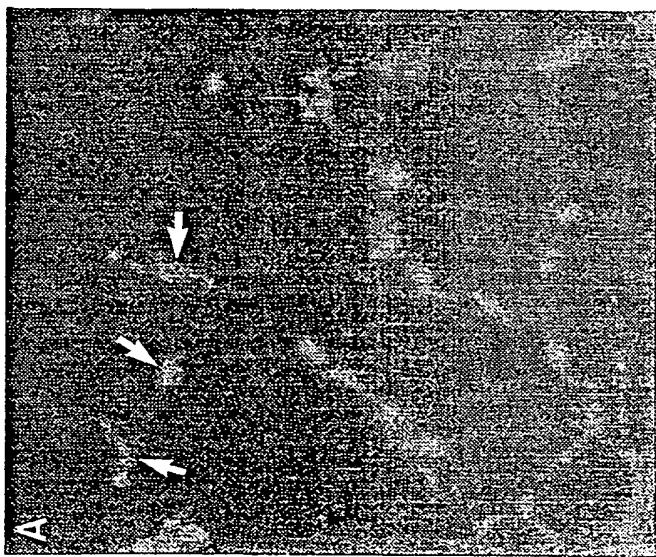
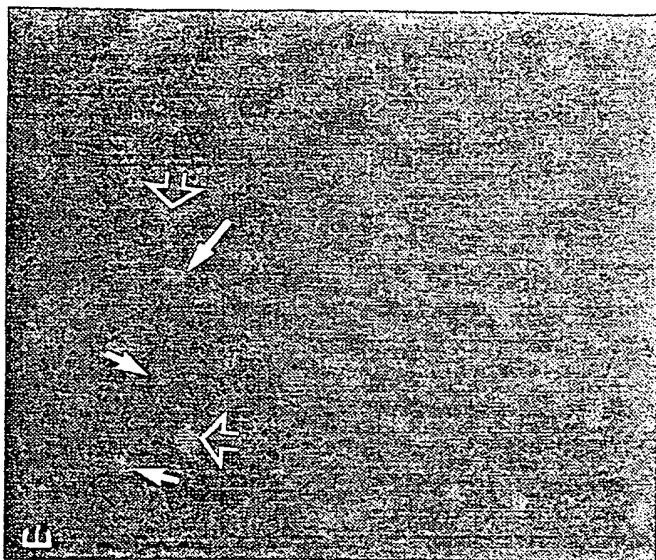
A**B**

Fig - 15

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E 16, 16

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL93/07000

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 5 C12N5/06 C12N5/08 C12N5/10 C12P21/08 G01N33/569
 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
 IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CELL vol. 71 , 11 December 1992 , CAMBRIDGE, NA US pages 973 - 985 DEREK L. STEMPLE ET AL. 'ISOLATION OF A STEM CELL FOR NEURONS AND GLIA FROM THE MAMMALIAN NEURAL CREST' see the whole document ---	1-43
X	WO,A,89 03872 (AMRAD CORPORATION LIMITED) 5 May 1989 see page 1, line 1 - page 5, line 29 ---	44-55
A	US,A,4 705 751 (DEANE F. MOSHER) 10 November 1987 see column 1, line 67 - column 2, line 12 ---	7,11 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US,A,4 707 448 (EUGENE O. MAJOR) 17 November 1987 see column 1, line 49 - line 68 -----	44

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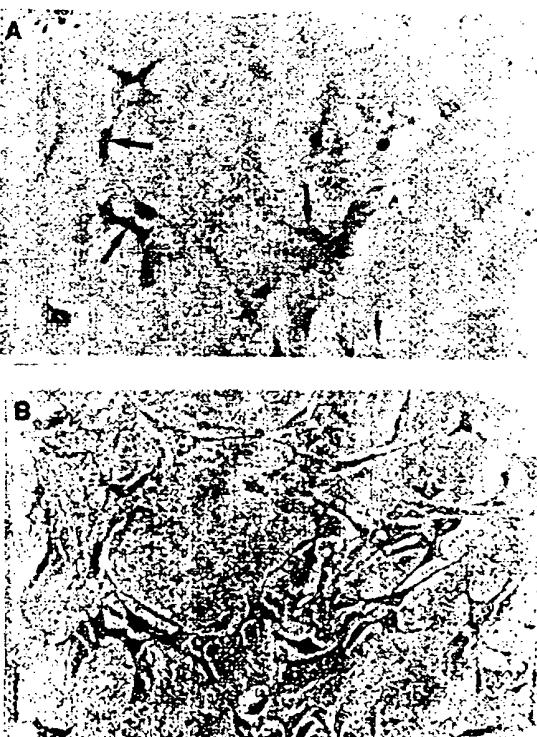
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/06, 5/08, 5/10 C12P 21/08, G01N 33/569, 33/577		A1	(11) International Publication Number: WO 94/02593 (43) International Publication Date: 3 February 1994 (03.02.94)
(21) International Application Number: PCT/US93/07000		(72) Inventors; and	
(22) International Filing Date: 26 July 1993 (26.07.93)		(75) Inventors/Applicants (for US only) : ANDERSON, David, J. [US/US]; 2891 North Mount Curve Avenue, Altadena, CA 91001 (US). STEMPLE, Derek, L. [US/US]; Cardiovascular Research Center, Massachusetts General Hospital East, 149 Thirteenth Street, 4th Floor, Charlestown, MA 02129 (US).	
(30) Priority data: 07/920,617 27 July 1992 (27.07.92) US 07/969,088 29 October 1992 (29.10.92) US		(74) Agents: TRECARTIN, Richard, F. et al.; Flehr, Hohbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).	
(60) Parent Applications or Grants (63) Related by Continuation US 07/969,088 (CIP) Filed on 29 October 1992 (29.10.92) US 07/920,617 (CIP) Filed on 27 July 1992 (27.07.92)		(81) Designated States: AU, CA, JP, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(71) Applicant (for all designated States except US): CALIFORNIA INSTITUTE OF TECHNOLOGY [US/US]; Office of Patents and Licensing, 1201 East California Boulevard, Pasadena, CA 91125 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: MAMMALIAN MULTIPOTENT NEURAL STEM CELLS

(57) Abstract

The invention includes mammalian multipotent neural stem cells and their progeny and methods for the isolation and clonal propagation of such cells. At the clonal level the stem cells are capable of self regeneration and asymmetrical division. Lineage restriction is demonstrated within developing clones which are sensitive to the local environment. The invention also includes such cells which are transfected with foreign nucleic acid, e.g., to produce an immortalized neural stem cell. The invention further includes transplantation assays which allow for the identification of mammalian multipotent neural stem cells from various tissues and methods for transplanting mammalian neural stem cells and/or neural or glial progenitors into mammals. A novel method for detecting antibodies to neural cell surface markers is disclosed as well as a monoclonal antibody to mouse LNGFR.



* (Referred to in PCT Gazette No. 10/1994, Section II)

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MAMMALIAN MULTIPOTENT NEURAL STEM CELLS

This is a continuation-in-part of U.S. patent application Serial No. 07/996,088, filed October 29, 1992 which is a continuation-in-part of U.S. patent application Serial No. 07/920,617, filed July 27, 1992.

5 Field of the Invention

The invention relates to the isolation, regeneration and use of mammalian multipotent neural stem cells and progeny thereof.

Background

10 The neural crest is a transient embryonic precursor population, whose derivatives include cells having widely different morphologies, characteristics and functions. These derivatives include the neurons and glia of the entire peripheral nervous system, melanocytes, cartilage and connective tissue of the head and neck, stroma of various secretory glands and cells in the outflow tract of the heart (for review, see Anderson, D.J. (1989) Neuron 3:1-12). Much of the knowledge of the developmental potential and fate of neural crest cells comes from studies in avian systems.
15 Fate maps have been established in aves and provide evidence that several different crest cell derivatives may originate from the same position along the neural
20

-2-

tube (Le Dourain, N.M. (1980) Nature 286:663-669). Schwann cells, melanocytes and sensory and sympathetic neurons can all derive from the truncal region of the neural tube. On the other hand, some derivatives were 5 found to originate from specific regions of the crest, e.g., enteric ganglia from the vagal and sacral regions. These studies also revealed that the developmental potential of the neural crest population at a given location along the neural tube is greater 10 than its developmental fate. This suggests that the new environment encountered by the migrating crest cells influences their developmental fate.

Single-cell lineage analysis *in vivo*, as well as clonal analysis *in vitro*, have reportedly shown that early 15 avian neural crest cells are multipotential during, or shortly after, their detachment and migration from the neural tube. In avian systems, certain clones derived from single neural crest cells in culture were reported to contain both catecholaminergic and pigmented cells 20 (Sieber-Blum, M. et al. (1980) Dev. Biol. 80:96-106). Baroffio, A. et al. (1988) Proc. Natl. Acad. Sci. USA 85:5325-5329, reported that avian neural crest cells from the cephalic region could generate clones which gave rise to highly heterogeneous progeny when grown on 25 growth-arrested fibroblast feeder cell layers.

In vivo demonstration of the multipotency of early neural crest cells was reported in chickens by Bronner-Fraser, M. et al. (1989) Neuron 3:755-766. Individual neural crest cells, prior to their migration from the 30 neural tube, were injected with a fluorescent dye. After 48 hours, the clonal progeny of injected cells were found to reside in many or all of the locations to which neural crest cells migrate, including sensory and sympathetic ganglia, peripheral motor nerves and the 35 skin. Phenotypic analysis of the labelled cells

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revealed that at least some neural crest cells are multipotent *in vivo*.

Following migration from the neural tube, these early multipotent crest cells become segregated into different sublineages, which generate restricted subsets of differentiated derivatives. The mechanisms whereby neural crest cells become restricted to the various sublineages are poorly understood. The fate of neural crest derivatives is known to be controlled in some way by the embryonic location in which their precursors come to reside (Le Douarin, N.M. (1982) The Neural Crest., Cambridge University Press, Cambridge, UK). The mechanism of specification for neural crest cells derivatives is not known. In culture studies described above, investigators reported that clones derived from primary neural crest cells exhibited a mixture of phenotypes (Sieber-Blum, M. et al. (1980) *ibid*; Baroffio, A. et al. (1988) *ibid*; Cohen, A.M. et al. (1975) Dev. Biol. 46:262-280; Dupin, E. et al. (1990) Proc. Natl. Acad. Sci. USA 87:1119-1123). Some clones contained only one differentiated cell type whereas other clones contained many or all of the assayable crest phenotypes.

The observation that apparently committed progenitors and multipotent cells coexist in the neural crest may be interpreted to reflect a pre-existing heterogeneity in the population of primary crest cells or it may reflect asynchrony in a population of cells that undergoes a progressive restriction in developmental potential. Given the uncertainty in the art concerning the developmental potential of neural crest cells, it is apparent that a need exists for the isolation of neural crest cells in clonal cultures. Although culture systems have been established which allow the growth and differentiation of isolated avian neural

crest cells thereby permitting phenotypic identification of their progeny, culture conditions which allow the self-renewal of multipotent mammalian neural crest cells have not been reported. Such 5 culture conditions are essential for the isolation of mammalian neural crest stem cells. Such stem cells are necessary in order to understand how multipotent neural crest cells become restricted to the various neural crest derivatives. In particular, culture conditions 10 which allow the growth and self-renewal of mammalian neural crest stem cells are desirable so that the particulars of the development of these mammalian stem cells may be ascertained. This is desirable because a number of tumors of neural crest derivatives exist in 15 mammals, particularly humans. Knowledge of mammalian neural crest stem cell development is therefore needed to understand these disorders in humans. Additionally, the ability to isolate and grow mammalian neural crest stem cells *in vitro* allows for the possibility of using 20 said stem cells to treat peripheral neurological disorders in mammals, particularly humans.

Accordingly, it is an object herein to provide clonal cultures of mammalian multipotent neural stem cells and their progeny in feeder cell-independent cultures. 25 Another object of the invention is directed to the demonstration that multipotential stem cells exist in the neural crest. Another object of the invention is the demonstration that these multipotent neural crest stem cells have at least limited self regeneration 30 capacity and undergo lineage restriction in a manner that is sensitive to the local environment.

A further object of the invention is to provide methods which allow the growth and regeneration of multipotent neural stem cells in feeder cell-independent cultures. 35 Another object of the invention is to provide methods

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which allow the differentiation of multipotent neural crest stem cells into at least the progenitors for, as well as, more differentiated neurons and glia of the peripheral nervous system (PNS). A further object of
5 the invention is to provide methods which allow for the identification of mammalian multipotent neural stem cells using transplantation assays. Still further, an object of the invention is to provide methods for transplanting neural crest stem cells or their progeny
10 into a mammal.

A further object of the invention is to extend the above methods to provide clonal cultures of mammalian neural crest stem cells and their progeny, to the detection or purification of glial or neuronal
15 progenitor cells, and to provide methods which allow the growth, regeneration and differentiation of such cells from tissues other than the embryonic neuronal crest. Still further, it is an object herein to provide methods for transplanting progenitors of such
20 glial and neuronal cells and multipotent stem cell precursor thereof into a mammal.

A further object of the invention is to provide cultures of genetically-engineered multipotent neural stem cells and their progeny. Still further, an object of the invention is to provide methods for the generation of cultures of such genetically-engineered
25 multipotent neural stem cells and their progeny including methods for immortalizing such cells.

Further, an object of the invention is to provide
30 monoclonal antibodies capable of recognizing surface markers which characterize multipotent neural stem cells and/or their progeny. A further object is to provide a novel procedure for screening sera and hybridomas for such antibodies.

Summary of the Invention

In accordance with the forgoing objects, the invention includes the isolation, clonal expansion and differentiation of mammalian multipotent neural stem cells such as those derived from the neural crest. The methods employ novel separation and culturing regimens and bioassays for establishing the generation of multipotent neural stem cells and their derivatives.

5 These methods result in the production of non-transformed neural stem cells and their progeny. The invention demonstrates, at the clonal level, the self regeneration and asymmetrical division of mammalian neural stem cells for the first time in feeder cell-independent cultures. Lineage restriction is

10 demonstrated within a developing clone and is shown to be sensitive to the local environment. For example, neural crest stem cells cultured on a mixed substrate of poly-D-lysine and fibronectin generate PNS neurons and glia, but on fibronectin alone the stem cells

15 generate PNS glia but not neurons. The neurogenic potential of the neural crest stem cells, while not expressed, is maintained over time on fibronectin. Therefore, both the overt differentiation and maintenance of a latent developmental potential of

20 neural crest stem cells are shown to be sensitive to the environment. The invention further includes transplantation assays which allow for the identification of mammalian multipotent neural stem cells from various tissues. It also includes methods

25 for transplanting mammalian neural stem cells and/or neural or glial progenitors into mammals.

The invention also provides methods for obtaining a cellular composition from mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neural progenitor cell or a multipotent stem cell precursor of such cells. The

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- method comprises preparing a suspension comprising a population of cells from a mammalian tissue; contacting the cell suspension with a culture medium and substrate which permits self-renewal of one or more of the glial 5 or neural progenitor cells or multipotent stem cell precursor, if present, in the cell suspension; and identifying one or more such cells by its ability to self-renew and differentiate feeder-cell independent culture.
- 10 The invention also includes alternate methods for obtaining a cellular composition comprising one or more cells having at least one property characteristic of a glial or neural progenitor cell or a multipotent stem cell precursor thereof. The method comprises preparing 15 a suspension comprising cells from a mammalian tissue; contacting the suspension with an antibody capable of forming a complex with a neural cell-specific surface marker on said glial or neural progenitor cells or multipotent stem cell precursor; and isolating the 20 complex, if formed, to obtain said cellular composition.

The invention is also directed to cells made according to any of the foregoing methods.

The invention also includes cultures of genetically-25 engineered mammalian multipotent neural stem cells and their progeny. Nucleic acid sequences encoding genes of interest are introduced into multipotent neural stem cells where they are expressed. These genes can include neurotrophic or survival factors and 30 immortalizing oncogenes. In addition, marker genes, such as the *E. coli* β -galactosidase gene, can be introduced to provide neural stem cells and their progeny which can be identified based on the expression of the marker gene. Selectable marker genes, such as

the neomycin phosphoribosyltransferase (neomycin-resistance, neo') or hisD genes, may be introduced to provide for a population of genetically-engineered stem cells which are identified by the ability to grow in
5 the presence of selective pressure (i.e., medium containing neomycin or L-histidinol). Neural stem cells may be transfected (genetically-engineered) with both a selectable marker and a non-selectable marker to provide neural stem cells which express both gene
10 products.

The invention also includes methods for producing cultures of genetically-engineered mammalian multipotent neural stem cells and their progeny.

Still further, the invention includes methods for immortalizing such cell lines by transfecting a glial or neural progenitor cell or multipotent stem cell precursor thereof with a vector comprising at least one immortalizing gene.
15

Further, the invention includes monoclonal antibodies capable of recognizing surface markers characteristic of mammalian multipotent neural stem cells and their progeny. The invention also includes a method for screening hybridoma producing such monoclonal antibodies which comprises contacting live neural cells
20 with monoclonal antibodies from a hybridoma and detecting whether the monoclonal antibody binds to the neural cell.
25

Brief Description of the Drawings

Figure 1A depicts the migration of rat neural crest
30 cells from the neural tube.

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Figure 1B demonstrates the expression of LNGFR and nestin by neural crest cells.

Figures 1C and 1D show the FACS profile from neural crest cells stained with anti-LNGFR (1D) and a control showing the background staining of the secondary antibody (1C).
5

Figure 2 demonstrates the clonal expansion of LNGFR⁺, nestin⁺ rat neural crest cells.

Figure 3 is a flow chart summarizing experiments 10 demonstrating the multipotency of mammalian neural crest cells.

Figure 4 demonstrates the expression of neuronal traits in clones derived from LNGFR⁺ founder cells.

Figure 5 demonstrates the expression of Schwann cell 15 phenotype by neural crest-derived glia.

Figure 6 shows the expression of peripherin, GFAP, and O₄ in a clone derived from a LNGFR⁺ founder cell.

Figure 7 is a flow chart summarizing experiments 20 demonstrating the self-renewal of mammalian neural crest cells.

Figure 8 demonstrates the self-renewal of multipotent neural crest cells.

Figure 9 demonstrates the multipotency of secondary founder cells.

25 Figure 10 provides a flow chart summarizing experiments demonstrating the substrate effect on the fate of mammalian neural crest cells.

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Figure 11 demonstrates that the neuronal differentiation of multipotent neural crest cells is affected by their substrate.

Figure 12 summarizes the percentage of different clone types which result when founder cells are grown on either FN or FN/PDL substrates.

Figure 13 provides a flow chart summarizing experiments demonstrating the instructive effect of the substrate on neural crest cell fate.

Figure 14 summarizes the percentage of the different clone types which result when founder cells are treated with a PDL lysine overlay at 48 hours (panel A) or day 5 (panel B).

Figure 15 demonstrates the genetic-engineering of a multipotent neural stem cell. Panel A depicts the expression of *E. coli* β -galactosidase (*lacZ*) in neural crest stem cells following infection with a *lacZ*-containing retrovirus. β -galactosidase⁺ cells are indicated by the solid arrows. Panel B depicts neural crest stem cells in phase contrast, in the same microscopic field as shown in Panel A. Cells which do not express β -galactosidase are indicated by open arrows.

Figure 16 demonstrates the specificity of a supernatant from a hybridoma culture producing monoclonal antibody specific to mouse LNGFR. Supernatants were screened using live Schwann cells isolated from mouse sciatic nerve. Panel A shows that most cells are stained with anti-LNGFR antibody (red staining; open arrows). Panel B shows Schwann cell nuclei counter stained with DAPI. Comparison with Panel A reveals a few cells not labeled by anti-LNGFR antibody (blue staining; open arrows).

Detailed Description of the Invention

The invention is directed, in part, to the isolation and clonal propagation of non-transformed mammalian neural crest stem cells and to multipotent neural stem cells from other embryonic and adult tissue. The invention also includes the production of neural crest stem cell and multipotent neural stem cell derivatives including progenitor and more differentiated cells of the neuronal and glial lineages. The invention is illustrated using neural crest stem cells isolated from the rat. The invention, however, encompasses all mammalian neural crest stem cells and multipotent neural stem cells and their derivatives and is not limited to neural crest stem cells from the rat.

Mammalian neural crest stem cells and multipotent neural stem cells and their progeny can be isolated from tissues from human and non-human primates, equines, canines, felines, bovines, porcines, lagomorphs, etc.

The invention encompasses several important methodological innovations: 1) the use of monoclonal antibodies to the low-affinity Nerve Growth Factor Receptor (LNGFR) as a cell surface marker to isolate and identify neural crest stem cells, a method extensible to other neural stem cell populations as well; 2) the development of cell culture substrates and medium compositions which permit the clonal expansion of undifferentiated neural crest cells; 3) the development of culture substrates and medium compositions which permit the differentiation of mammalian neural crest cells into their differentiated derivatives (including but not restricted to peripheral neurons and glia) in clonal culture.

The invention also provides neural crest stem cells and other multipotent neural stem cells. It is important

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to understand that such cells could not be identified as stem cells without the development of the isolation and cell culture methodologies summarized above. The identification of a neural stem cell requires that 5 several criteria be met: 1) that the cell be an undifferentiated cell capable of generating one or more kinds of differentiated derivatives; 2) that the cell have extensive proliferative capacity; 3) that the cell be capable of self-renewal or self-maintenance (Hall et 10 al. (1989) Development 106:619; Potten et al. (1990) Crypt. Development 110:1001). The concept of a stem cell as obligatorily capable of "unlimited" self-renewal is applicable only to regenerating tissues such as skin or intestine. In the case of a developing 15 embryo stem cells may have limited self-renewal capacity but be stem cells nevertheless (Potten et al. (1990) *supra*). The development of clonal culture methods permitted the demonstration of criteria 1 and 2 herein. The development of sub-clonal culture 20 methods (i.e., the ability to clone single neural stem cells, and then re-clone progeny cells derived from the original founder cell) further permitted the demonstration herein of criterion 3.

To appreciate the significance of this demonstration, 25 consider an alternative hypothesis for cells from the neural crest: individual undifferentiated neural crest cells divide to generate both neurons and glia (i.e., meet criteria 1 and 2 above), but the daughter cells produced by these initial cell divisions are committed 30 to producing either neurons or glia, but not both. In this case, the neural crest cell is a progenitor cell but not a stem cell, because it does not have self-renewal capacity. If this were the case, then upon sub-cloning of neural crest cell clones, the resulting 35 "secondary" clones could contain either neurons or glia, but not both. This is not observed. Rather,

- most or all of the secondary clones contain both neurons and glia, like their parent clones. This experiment thus provides the first definitive evidence that neural progenitor cells from any region of the nervous system have stem cell properties. In no other set of published experiments have these stringent criteria for stem cell properties been met, despite claims that "stem cells" have been isolated or identified (Cattaneo et al. (1991) Trends Neurosci. 14:338; Reynolds et al. (1992) Science 255:1707) from the mammalian central nervous system. This in part reflects imprecise use of the term "stem cell" and in part the failure to perform adequate experimental tests to support the existence of such cells.
- As used herein, the term "non-transformed cells" means cells which are able to grow *in vitro* without the need to immortalize the cells by introduction of a virus or portions of a viral genome containing an oncogene(s) which confers altered growth properties upon cells by virtue of the expression of viral genes within the transformed cells. These viral genes typically have been introduced into cells by means of viral infection or by means of transfection with DNA vectors containing isolated viral genes.
- As used herein, the term "genetically-engineered cell" refers to a cell into which a foreign (*i.e.*, non-naturally occurring) nucleic acid, *e.g.*, DNA, has been introduced. The foreign nucleic acid may be introduced by a variety of techniques, including, but not limited to, calcium-phosphate-mediated transfection, DEAE-mediated transfection, microinjection, retroviral transformation, protoplast fusion and lipofection. The genetically-engineered cell may express the foreign nucleic acid in either a transient or long-term manner.
- In general, transient expression occurs when foreign

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DNA does not stably integrate into the chromosomal DNA of the transfected cell. In contrast, long-term expression of foreign DNA occurs when the foreign DNA has been stably integrated into the chromosomal DNA of
5 the transfected cell.

As used herein, an "immortalized cell" means a cell which is capable of growing indefinitely in culture due to the introduction of an "immortalizing gene(s)" which confers altered growth properties upon the cell by
10 virtue of expression of the immortalizing gene(s) within the genetically engineered cell. Immortalizing genes can be introduced into cells by means of viral infection or by means of transfection with vectors containing isolated viral nucleic acid encoding one or
15 more oncogenes. Viruses or viral oncogenes are selected which allow for the immortalization but preferably not the transformation of cells. Immortalized cells preferably grow indefinitely in culture but do not cause tumors when introduced into
20 animals.

As used herein, the term "transformed cell" refers to a cell having the properties of 1) the ability to grow indefinitely in culture and 2) causing tumors upon introduction into animals. "Transformation" refers to
25 the generation of a transformed cell.

As used herein, the term "feeder-cell independent culture" or grammatical equivalents means the growth of cells *in vitro* in the absence of a layer of different cells which generally are first plated upon a culture dish to which cells from the tissue of interest are added. The "feeder" cells provide a substratum for the attachment of the cells from the tissue of interest and additionally serve as a source of mitogens and survival factors. The feeder-cell independent cultures herein
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utilize a chemically defined substratum, for example fibronectin (FN) or poly-D-lysine (PDL) and mitogens or survival factors are provided by supplementation of the liquid culture medium with either purified factors or
5 crude extracts from other cells or tissues. Therefore, in feeder-cell independent cultures, the cells in the culture dish are primarily cells derived from the tissue of interest and do not contain other cell types required to support the growth of the cells derived
10 from the tissue of interest.

As used herein, the term "clonal density" means a density sufficiently low enough to result in the isolation of single, non-impinging cells when plated in a culture dish, generally about 225 cells/100 mm
15 culture dish.

As used herein, the term "neural crest stem cell" means a cell derived from the neural crest which is characterized by having the properties (1) of self-renewal and (2) asymmetrical division; that is, one
20 cell divides to produce two different daughter cells with one being self (renewal) and the other being a cell having a more restricted developmental potential, as compared to the parental neural crest stem cell. The foregoing, however, is not to be construed to mean
25 that each cell division of a neural crest stem cell gives rise to an asymmetrical division. It is possible that a division of a neural crest stem cell can result only in self-renewal, in the production of more developmentally restricted progeny only, or in the
30 production of a self-renewed stem cell and a cell having restricted developmental potential.

As used herein, the term "multipotent neural stem cell" refers to a cell having properties similar to that of a neural crest stem cell but which is not necessarily

derived from the neural crest. Rather, as described hereinafter, such multipotent neural stem cells can be derived from various other tissues including neural epithelial tissue from the brain and/or spinal cord of the adult or embryonic central nervous system or neural epithelial tissue which may be present in tissues comprising the peripheral nervous system. In addition, such multipotent neural stem cells may be derived from other tissues such as lung, bone and the like utilizing the methods disclosed herein. It is to be understood that such cells are not limited to multipotent cells but may comprise a pluripotent cell capable of regeneration and differentiation to different types of neurons and glia, e.g., PNS and CNS neurons and glia or progenitors thereof. In this regard, it should be noted that the neural crest stem cells described herein are at least multipotent in that they are capable, under the conditions described, of self-regeneration and differentiation to some but not all types of neurons and glia *in vitro*. Thus, a neural crest stem cell is a multipotent neural stem cell derived from a specific tissue, i.e., the embryonic neural tube.

In most embodiments, neural crest stem cells are further characterized by a neural cell-specific surface marker. Such surface markers in addition to being found on neural crest stem cells may also be found on other multipotent neural stems derived therefrom, e.g., glial and neuronal progenitor cells of the peripheral nervous system (PNS) and central nervous system (CNS). An example is the cell surface expression of a nerve growth factor receptor on neural crest stem cells. In rat, humans and monkeys this nerve growth factor receptor is the low-affinity nerve growth factor receptor (LNGFR). Such stem cells may also be characterized by the expression of nestin, an intracellular intermediate filament protein. Neural

crest stem cells may be further characterized by the absence of markers associated with mature PNS neuronal or glial cells. In the rat, such markers include sulfatide, glial fibrillary acidic protein (GFAP) and myelin protein P₀ in PNS glial cells and peripherin and neurofilament in PNS neuronal cells.

LNGFR is a receptor for nerve growth factor, a neurotrophic factor shown to be responsible for neuronal survival in vivo. LNGFR is found on several mammalian cell types including neural crest cells and Schwann cells (glial cells of the PNS) as well as on the surface of cells in the ventricular zone throughout the embryonic central nervous systems. (See, e.g., Yan et al. (1988) J. Neurosci. 8:3481-3496 and Heuer, J.G et al. (1980) Neuron 5:283-296 which studied such cells in the rat and chick systems, respectively.) Antibodies specific for LNGFR have been identified for LNGFR from rat monoclonal antibodies 217c (Peng, W.W. et al. (1982) Science 215:1102-1104) and 192-Ig (Brockes, J.P. et al. (1977) Nature 266:364-366 and Chandler, C.E. et al. (1984) J. Biol. Chem. 259:6882-6889) and human (Ross, A.H. et al. (1984) Proc. Natl. Acad. Sci. USA 81:6681-6685; Johnson, et al. (1986) Cell 47:545-554; Loy et al. (1990) J. Neurosci Res. 27:651-644). The monoclonal antibody against human LNGFR has been reported to cross-react with LNGFR from monkeys (Mufson, E.G. et al. (1991) J. Comp. Neurol. 308:555-575). The DNA sequence has been determined for rat and human LNGFR (Radeke, M.J. et al. (1987) Nature 325:593-597 and Chao, M.V. et al. (1986) Science 232:518-521, respectively) and is highly conserved between rat and human.

Using the following techniques, monoclonal antibodies specific for LNGFR from any desired mammalian species 35 are generated by first isolating the nucleic acid

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encoding the LNGFR protein. One protocol for obtaining such nucleic acid sequences uses one or more nucleic acid sequences from a region of the LNGFR gene which is highly conserved between mammalian species, e.g., rat
5 and human, as a hybridization probe to screen a genomic library or a cDNA library derived from mammalian tissue from the desired species (Sambrook, J. et al. (1989) Cold Spring Harbor Laboratory Press. Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 8.3-8.80,
10 9.47-9.58 and 11.45-11.55). The cloned LNGFR sequences are then used to express the LNGFR protein or its extracellular (ligand binding) domain in an expression host from which the LNGFR protein is purified. Purification is performed using standard techniques
15 such as chromatography on gel filtration, ion exchange or affinity resins. The purified LNGFR is then used to immunize an appropriate animal (e.g., mouse, rat, rabbit, hamster) to produce polyclonal antisera and to provide spleen cells for the generation of hybridoma
20 cell lines secreting monoclonal antibodies specific for LNGFR of the desired species (Harlow, E. et al. (1988) Cold Spring Harbor Laboratory Press, Antibodies: A Laboratory Manual, pp. 139-242).

A novel screening method can be used to detect the
25 production of antibody against LNGFR or any other surface marker which characterizes a multipotent neural stem cell or progeny thereof. The method can be practiced to detect animals producing polyclonal antibodies against a particular antigen or to identify
30 and select hybridomas producing monoclonal antibodies against such antigens. In this method, serum from an immunized animal or supernatent from a hybridoma culture is contacted with a live neural cell which displays a surface marker characteristic of a
35 particular neural cell line. Detection of whether binding has occurred or not is readily determined by

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any number of known methods. A particularly preferred method is to use labeled antibody which is specific for the immunoglobulins produced by the species which is immunized with the particular antigen and which is a source for polyclonal serum and spleen cells for hybridoma formation.

The live neural cell used in the foregoing antibody assay is dependent upon the particular surface marker for which an antibody is desired. In the examples, a monoclonal antibody for mouse LNGFR was identified using a dissociated primary culture of Schwann cells. In conjunction with the assay disclosed in the examples, mouse fibroblasts acted as a negative control. However, primary cultures of other cell lines can be used to detect monoclonal antibodies to LNGFR. For example, forebrain cholinergic neurons or sensory neurons can be used. In addition, a primary culture of epithelial cells can be used as a negative control.

Other markers found on neural cells include Platelet Derived Growth Factor Receptor (PDGFR), Fibroblast Growth Factor (FGF) and Stem Cell Factor Receptor (SCFR). Cells useful for detecting monoclonal antibodies to PDGFR and FGF include primary cultures of glial cells or fibroblasts. Negative controls include cultures of epithelial cells and neuroblastomas. SCFR is expressed on a subset of neuronal cells. Primary cultures of melanocytes or melanoma cells can be used to detect monoclonal antibodies to this receptor. Negative controls include primary cultures of fibroblasts and glial cells.

It is not always necessary to generate polyclonal or monoclonal antibodies that are species specific. Monoclonal antibodies against an antigenic determinant from one species may react against that antigen from

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more than one species. For example, as stated above, the antibody directed against the human LNGFR molecule also recognizes LNGFR on monkey cells. When cross-reactive antibodies are available, there is no need to 5 generate antibodies which are species specific using the methods described above.

Nestin, a second marker in the neural crest stem cell, is an intermediate filament protein primarily located intracellularly, which has been shown to be present in 10 CNS neuroepithelial cells and Schwann cells in the peripheral nervous system of rats (Friedman et al. (1990) J. Comp. Neurol. 295:43-51). Monoclonal antibodies specific for rat nestin have been isolated: Rat 401, (Hockfield, S. et al. (1985) J. Neurosci. 15 5(12):3310-3328). A polyclonal rabbit anti-nestin antisera has been reported which recognizes mouse nestin (Reynolds, D.A. et al. (1992) Science 255:1707- 20 1710). The DNA sequences encoding the rat nestin gene have been cloned (Lendahl, U. et al. (1990) Cell 60:585-595). These DNA sequences are used to isolate nestin clones from other mammalian species. These DNA sequences are then used to express the nestin protein and monoclonal antibodies directed against various 25 mammalian nestins are generated as described above for LNGFR.

As used herein, the term "glial progenitor cell" refers to a cell which is intermediate between the fully differentiated glial cell and a precursor multipotent neural stem cell from which the fully differentiated 30 glial cell develops. In general, such glial progenitor cells are derived according to the methods described herein for isolating such cells from various tissues including adult and embryonic CNS and PNS tissue as well as other tissues which may potentially contain 35 such progenitors.

As used herein, the term "PNS glial progenitor cell" means a cell which has differentiated from a mammalian neural crest stem cell which is committed to the PNS glial lineage and is a dividing cell but does not yet express surface or intracellular markers found on more differentiated, non-dividing PNS glial cells. Such progenitor cells are preferably obtained from neural crest stem cells isolated from the embryonic neural crest which have undergone further differentiation.

5 However, equivalent cells may be derived from other tissue. When PNS glial progenitor cells are placed in appropriate culture conditions they differentiate into PNS glia expressing the appropriate differentiation markers, for example, sulfatide and GFAP.

10

15 Sulfatide is a glycolipid molecule found on the surface of Schwann cells and oligodendrocytes in rats, mice, chickens and humans. The expression of sulfatide on Schwann cells is dependent upon either axonal contact or exposure to cyclic AMP or analogs thereof, such as

20 forskolin (Mirsky, R. et al. (1990) Development 109:105-116). Monoclonal antibodies specific for sulfatide have been reported (Sommer, I. et al. (1981) Dev. Biol. 83:311-327).

25

30 Glial fibrillary acidic protein (GFAP) is an intermediate filament protein specifically expressed by astrocytes and glial cells of the CNS and by Schwann cells, the glial cells of the PNS (Jessen, K.R. et al. (1984) J. Neurocytology 13:923-934 and Fields, K.L. et al. (1989) J. Neuroimmuno. 8:311-330). Monoclonal antibodies specific for GFAP have been reported (Debus et al. (1983) Differentiation 25:193-203). Mouse and human GFAP genes have been cloned (Cowan, N.J. et al. (1985) N.Y. Acad. Sci. 455:575-582 and Bongcamrudlowss, D. et al. (1991) Cancer Res. 51:1553-1560,

35 respectively). These DNA sequences are used to isolate

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GFAP clones from other mammalian species. These DNA sequences are then used to express the GFAP protein and monoclonal antibodies directed against various mammalian GFAPs are generated as described above for
5 LNGFR.

As used herein, the term "factors permissive for PNS glial cell differentiation" means compounds, such as, but not limited to, protein or steroid molecules or substrates such as FN or PDL, which permit at least
10 neural crest stem cells to become restricted to the PNS glial lineage. Such lineage-restricted progeny of neural crest stem cells include glial progenitor cells, which are at least bipotential, in that they can divide to give rise to self, as well as, more mature non-
15 dividing PNS glial cells.

As used herein, the term "neuronal progenitor cell" refers to a cell which is intermediate between the fully differentiated neuronal cell and a precursor multipotent neural stem cell from which the fully
20 differentiated neuronal cell develops. In general, such neuronal progenitor cells are derived according to the methods described herein for isolating such cells from various tissues including adult and embryonic CNS and PNS tissue as well as other tissues which may
25 potentially contain such progenitors.

As used herein, the term "PNS neuronal progenitor cell" means a cell which has differentiated from a mammalian neural crest stem cell which is committed to one or more PNS neuronal lineages and is a dividing cell but
30 does not yet express surface or intracellular markers found on more differentiated, non-dividing PNS neuronal cells. Such progenitor cells are preferably obtained from neural crest stem cells isolated from the embryonic neural crest which have undergone further

differentiation. However, equivalent cells may be derived from other tissue. When PNS neuronal progenitor cells are placed in appropriate culture conditions they differentiate into mature PNS neurons expressing the appropriate differentiation markers, for example, peripherin, neurofilament and high-polysialic acid neural cell adhesion molecule (high PSA-NCAM).

Peripherin, a 57 kDa intermediate filament protein, is expressed in adult rodents primarily in peripheral neurons. More limited expression of peripherin is found in some motoneurons of the spinal cord and brain stem and a limited group of CNS neurons. Peripherin is expressed in rat embryos primarily in neurons of peripheral ganglia and in a subset of ventral and lateral motoneurons in the spinal cord (Gorham, J.D. et al. (1990) Dev. Brain Res. 57:235-248). Antibodies specific for this marker have been identified in the rat (Portier, M. et al. (1983/84) Dev. Neurosci. 6:335-344). The DNA sequences encoding the rat peripherin gene have been cloned (Thompson, M.A. et al. (1989) Neuron 2:1043-1053). These DNA sequences are used to isolate DNA sequences for the peripherin gene in other mammals that are used to express the protein and generate antibodies directed against other mammalian peripherin proteins, as described above for LNGFR.

Neurofilaments are neuron-specific intermediate filament proteins. Three neurofilament (NF) proteins have been reported: NF68, a 68 kD protein also called NF-L (Light); NF160, a 160 kD protein also called NF-M (Medium); NF200, a 200 kD protein also called NF-H (Heavy). In general, there is coordinate expression of all three NF proteins in neurons. The DNA sequences encoding the rat NF200 and NF160 proteins have been cloned (Dautigny, A. et al. (1988) Biochem. Biophys. Res. Commun. 154:1099-1106 and Napolitano, E.W. et al.

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- (1987) J. Neurosci. 7:2590-2599, respectively). All three NF protein genes have been cloned in mice and humans. Mouse NF68 nucleic acid sequences were reported in Lewis, S.A. et al. (1985) J. Cell Biol. 100:843-850.
- 5 Mouse NF160 nucleic acid sequences were reported in Levy, E. et al. (1987) Eur. J. Biochem. 166:71-77. Mouse NF200 nucleic acid sequences were reported in Shneidman, P.S. et al. (1988) Mol. Brain Res. 4:217-231. In humans, nucleic acid sequences were reported
- 10 for: NF68, Julien, J.-P. et al. (1987) Biochem. Biophys. Acta. 909:10-20; NF160, Myers, M.W. et al. (1987) EMBO J. 6:1617-1626; NF200, Lee, J.F. et al. (1988) EMBO J. 7:1947-1955. These DNA sequences are used to produce the protein for the production of
- 15 antibodies or to isolate other mammalian NF genes and the proteins expressed and antibodies generated for any desired species, as described above for LNGFR. As used herein, the term "NF⁺" means expression of one or more of the three NF proteins.
- 20 As used herein, the term "factors permissive for PNS neuronal cell differentiation" means compounds, such as, but not limited to, protein or steroid molecules or substrates such as FN or PDL, which permit at least a neural crest stem cell to become restricted to the PNS neuronal lineage. Such lineage-restricted progeny of neural crest stem cells include PNS neuronal progenitor cells, which are at least bipotential, in that they can divide to give rise to self, as well as, more mature, non-dividing PNS neurons.
- 25 Mammalian neural crest stem cell compositions are provided which serve as a source for neural crest cell derivatives such as neuronal and glial progenitors of the PNS which in turn are a source of PNS neurons and glia. Methods are provided for the isolation and
- 30 clonal culture of neural crest stem cells, in the
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absence of feeder cells. In the examples provided, these methods utilize a chemically defined medium which is supplemented with chick embryo extract as a source of mitogens and survival factors. Factors present in
5 the extract of chicken embryos allow the growth and self renewal of rat neural crest stem cells. However, media used to isolate and propagate rat neural crest stem cells can be used to isolate and propagate neural crest stem cells from other mammalian species, such as
10 human and non-human primates, equines, felines, canines, bovines, porcines, lagomorphs, etc.

Culture conditions provided herein allow the isolation self-renewal and differentiation of mammalian neural crest stem cells and their progeny. These culture
15 conditions may be modified to provide a means of detecting and evaluating growth factors relevant to mammalian neural crest stem cell self-renewal and the differentiation of the stem cell and its progeny. These modifications include, but are not limited to,
20 changes in the composition of the culture medium and/or the substrate and in the specific markers used to identify either the neural crest stem cell or their differentiated derivatives.

Culture conditions are provided which allow the
25 differentiation of mammalian neural crest stem cells into the PNS neuronal and glial lineages in the absence of feeder cell layers. In addition to liquid culture media, these culture conditions utilize a substratum comprising fibronectin alone or in combination with
30 poly-D-lysine. In the examples provided, human fibronectin is utilized for the culturing of rat neural crest stem cells and their progeny. Human fibronectin can be used for the culturing of neural crest stem cells isolated from avian species as well as from any
35 mammal, as the function of the fibronectin protein is

highly conserved among different species. Cells of many species have fibronectin receptors which recognize and bind to human fibronectin.

In order to isolate the subject neural crest stem 5 cells, it is necessary to separate the stem cell from other cells in the embryo. Initially, neural crest cells are obtained from mammalian embryos.

For isolation of neural crest cells from mammalian embryos, the region containing the caudal-most 10 somites are dissected from early embryos (equivalent to gestational day 10.5 day in the rat). These trunk sections are transferred in a balanced salt solution to chilled depression slides, typically at 4°C, and treated with collagenase in an appropriate buffer 15 solution such as Howard's Ringer's solution. After the neural tubes are free of somites and notochords, they are plated onto fibronectin (FN)-coated culture dishes to allow the neural crest cells to migrate from the neural tube. Twenty-four hours later, following 20 removal of the tubes with a sharpened tungsten needle, the crest cells are removed from the FN-coated plate by treatment with a Trypsin solution, typically at 0.05%. The suspension of detached cells is then collected by centrifugation and plated at an appropriate density, 25 generally 225 cells/100mm dish in an appropriate chemically defined medium. This medium is preferentially free of serum and contains components which permit the growth and self-renewal of neural crest stem cells. The culture dishes are coated with 30 an appropriate substratum, typically a combination of FN and poly-D-lysine (PDL).

Procedures for the identification of neural crest stem cells include incubating cultures of crest cells for a short period of time, generally 20 minutes, at room

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temperature, generally about 25°C, with saturating levels of antibodies specific for a particular marker, e.g., LNGFR. Excess antibody is removed by rinsing the plate with an appropriate medium, typically L15 medium (Gibco) supplemented with fresh vitamin mix and bovine serum albumin (L-15 Air). The cultures are then incubated at room temperature with a fluorochrome labelled secondary antibody, typically Phycoerythrin R-conjugated secondary antibody (TAGO) at an appropriate dilution for about 20 minutes. Excess secondary antibodies are then removed using an appropriate medium, such as L-15 Air. The plates are then covered with the chemically defined growth medium and examined with a fluorescence microscope. Individual LNGFR⁺ clones are isolated by fluorescence activated cell sorting (FACS) or, more typically, by marking the plate under the identified clone. The markings are typically made to a diameter of 3-4 mm, which generally allows for the unambiguous identification of the progeny of the founder cell at any time during an experiment. If desired, individual LNGFR⁺ clones are removed from the original plate by trypsinization with the use of cloning cylinders.

Procedures for permitting the differentiation of stem cells include the culturing of isolated stem cells in a medium permissive for differentiation to a desired lineage, such as Schwann cell differentiation (SCD) medium. Other procedures include growth of isolated stem cells on substrates capable of permitting differentiation, such as FN or FN and PDL.

Procedures for the serial subcloning of stem cells and their derivatives include the trypsinization of individual clones, as described above, followed by replating the clone on a desired substrate and culturing in a desired medium, such as a chemically

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defined medium suitable for maintenance of stem cells or SCD medium permissive for the differentiation of said neural crest stem cells. Crest cells may be identified following serial subcloning by live-cell 5 labeling with an antibody directed against LNGFR, as described above.

The methods described herein provide the basis of functional assays which allow for the identification and production of cellular compositions of mammalian 10 cells which have properties characteristic of neural crest stem cells, glial or neuronal progenitor cells or multipotent stem cell precursor of such progenitor cells. In order to isolate such cells from tissues other than embryonic neural tubes, it is necessary to 15 separate the progenitor and/or multipotent stem cells from other cells in the tissue. The methods presented in the examples for the isolation of neural crest stem cells from neural tubes can be readily adapted for other tissues by one skilled in the art. First, a 20 single cell suspension is made from the tissue; the method used to make this suspension will vary depending on the tissue utilized. For example, some tissues require mechanical disruption of the tissue while other tissues require digestion with proteolytic enzymes 25 alone or in combination with mechanical disruption in order to create the single cell suspension. Tissues such as blood already exists as a single cell suspension and no further treatment is required to generate a suspension, although hypotonic lysis of red 30 blood cells may be desirable. Once the single cell suspension is generated it may be enriched for cells expressing LNGFR or other neural cell-specific markers on their surface. One protocol for the enrichment for LNGFR⁺ cells is by incubating the cell suspension with 35 antibodies specific for LNGFR and isolating the LNGFR⁺ cells. Enrichment for cells expressing a neural cell-

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specific surface marker is particularly desirable when these cells represent a small percentage (less than 5%) of the starting population. The isolation of cells which have complexed with an antibody for a neural 5 cell-specific surface marker such as is carried out using any physical method for isolating antibody-labeled cells. Such methods include fluorescent-activated cell sorting in which case the cells, in general, are further labeled with a fluorescent 10 secondary antibody that binds the anti-LNGFR antibody, e.g., mouse anti-LNGFR and fluorescein label goat anti-mouse IgG; panning in which case the antibody-labeled cells are incubated on a tissue-culture plate coated 15 with a secondary antibody; Avidin-sepharose chromatography in which the anti-LNGFR antibody is biotinylated prior to incubation with the cell suspension so that the complexed cells can be recovered on an affinity matrix containing avidin (i.e., where the antibody is an antibody conjugate with one of the 20 members of a binding pair); or by use of magnetic beads coated with an appropriate anti-antibody so that the labeled LNGFR-expressing cells can be separated from the unlabeled cells with the use of a magnet. All of the foregoing cell isolation procedures are standard 25 published procedures that have been used previously with other antibodies and other cells.

The use of antibodies specific for neural stem cell-specific surface markers results in the isolation of multipotent neural stem cells from tissues other than 30 embryonic neural tubes. For example, as previously indicated, LNGFR is expressed in cells of the ventricular zone throughout the embryonic central nervous system of the rat and chick. This implies that other mammalian species have a similar pattern of LNGFR 35 expression and studies in human with monoclonal antibodies against the human LNGFR (Loy, et al. (1990)

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J. Neurosci. Res. 27:651-654) are consistent with this expectation. Since cells from the ventricular zone (Cattaneo et al. (1991) Trends Neurosci. 14:338-340; Reynolds et al. (1992) Science 255:1707-1710) are
5 likely to be stem cells (Hall et al. (1989) Development 106:619-633; Potter et al. (1990) Development 110:1001-1020) antibodies to neural cell-specific surface markers should prove useful in isolating multipotent neural stem cells from the central and peripheral
10 nervous systems and from other tissue sources.

Alternatively, or in conjunction with the above immuno-isolation step, the cells are plated at clonal density, generally 225 cells/100 mm dish, in an appropriate chemically defined medium on a suitable substrate as
15 described in the examples for isolation of rat neural crest stem cells. The presence of neural crest-like stem cells (e.g., a multipotent neural stem cell) is confirmed by demonstrating that a single cell can both self-renew and differentiate to members of at least the
20 PNS neuronal and glial lineages utilizing the culture conditions described herein. Other types of multipotent neural stem cells are identified by differentiation to other cell type such as CNS neural or glial cells or their progenitors. Depending upon
25 the source of the tissue used in the foregoing methods, multipotent neural stem cells may not be obtained. Rather, further differentiated cell types such as glial and neuronal progenitor cells may be obtained.

Transplantation assay systems described herein provide
30 the basis of functional assays which allow for the identification of mammalian cells which have properties characteristic of neural crest stem cells, multipotent neural stem cells and/or neuronal or glial progenitor cells. Cells of interest, identified by either the *in vivo* or *in vitro* assays described above, are
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transplanted into mammalian hosts using standard surgical procedures. The transplanted cells and their progeny are distinguished from the host cells by the presence of species specific antigens or by the expression of an introduced marker gene. The transplanted cells and their progeny are also stained for markers of mature neurons and glia in order to examine the developmental potential of the transplanted cells. This transplantation assay provides a means to identify neural crest stem cells by their functional properties in addition to the *in vitro* culture assays described above.

Additionally, the transplantation of cells having characteristics of multipotent neural stem cells, neural crest stem cells or progenitors of neuronal or glial cells provides a means to investigate the therapeutic potential of these cells for neurological disorders of the PNS and CNS in animal models. Examples of PNS disorders in mice include the *trembler* and *shiverer* strains. The *trembler* mutation is thought to involve a defect in the structural gene for myelin basic protein (MBP). This mutation maps to the same region of chromosome 11 as does the MBP gene. This mutation results in the defective myelination of axons in the PNS. An analogous disorder is seen in humans, Charcot-Marie-Tooth syndrome, which results in progressive neuropathic muscular atrophy.

The *shiverer* mutation in mice results in a severe myelin deficiency throughout the CNS and a moderate hypo-myelination in the PNS. Severe shivering episodes are seen 12 days after birth. An analogous disorder is seen in humans, Guillaum-Barre' disease, which is characterized by an acute febrile polyneuritis.

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Cells having characteristics of multipotent neural stem cells, neural crest stem cells or neuronal or glial progenitors of the PNS or CNS (identified by either *in vitro* or *in vivo* assays) are introduced into a mammal exhibiting a neurological disorder to examine the therapeutic potential of these cells. These cells are preferably isolated from a mammal having similar MHC genotypes or the host mammal is immunosuppressed using drugs such as cyclosporin A. The cells are injected into an area containing various peripheral nerves known to be effected in a particular mammal or into the spinal cord or brain for mammals which show involvement of the CNS. The cells are injected at a range of concentrations to determine the optimal concentration into the desired site. Alternatively, the cells are introduced in a plasma clot or collagen gel to prevent rapid dispersal of cells from the site of injection. The effect of this treatment on the neurological status of the model animal is noted. Desired therapeutic effects in the above mutant mice include the reduction or cessation of seizures or improved movement of lower motor extremities.

There is strong interest in identifying the multipotent neural stem cells such as the neural crest stem cell and defining culture conditions which allow the clonal propagation and differentiation of said stem cells. Having possession of a multipotent neural stem cell or a neural crest stem cell allows for identification of growth factors associated with self regeneration. In addition, there may be as yet undiscovered growth factors associated with (1) with the early steps of restriction of the stem cell to a particular lineage; (2) the prevention of such restriction; and (3) the negative control of the proliferation of the stem cell or its derivatives.

The multipotent neural stem cell, neural crest stem cell, progeny thereof or immortalized cell lines derived therefrom are useful to: (1) detect and evaluate growth factors relevant to stem cell regeneration; (2) detect and isolate ligands, such as growth factors or drugs, which bind to receptors expressed on the surface of such cells or their differentiated progeny (e.g., Glial Growth Factor (GGF), Heregulin and Neu Differentiation Factor (NDF)); (3) provide a source of cells which express or secrete growth factors specific to multipotent neural stem cells; (4) detect and evaluate other growth factors relevant to differentiation of stem cell derivatives, such as neurons and glia; (5) produce various neural stem cell derivatives, including both the progenitors and mature cells of a given lineage and (6) provide a source of cells useful for treating neurological diseases of the PNS and CNS in model animal systems and in humans. The culture conditions used herein allow for the growth and differentiation of stem cells *in vitro* and provide a functional assay whereby mammalian tissues can be assayed for the presence of cells having the characteristics of neural stem cells. The transplantation assay described herein also provides a functional assay whereby mammalian neural stem cells may be identified.

As indicated in the examples, neural crest stem cells have been passaged for at least six-ten generations in culture. Although it may be unnecessary to immortalize those or other multipotent neural stem cell lines or progenitor cell lines obtained by the methods described herein, once a cell line has been obtained it may be immortalized to yield a continuously growing cell line useful for screening trophic or differentiation factors or for developing experimental transplantation therapies in animals. Such immortalization can be

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obtained in multipotent neural stem cells or progenitors of glial and neuronal cells by genetic modification of such cells to introduce an immortalizing gene.

- 5 Examples of immortalizing genes include: (1) nuclear oncogenes such as *v-myc*, *N-myc*, *T antigen* and *Ewing's sarcoma oncogene* (Fredericksen et al. (1988) Neuron 1:439-448; Bartlett, P. et al. (1988) Proc. Natl. Acad. Sci. USA 85:3255-3259, and Snyder, E.Y. et al. (1992) Cell 68:33-51), (2) cytoplasmic oncogenes such as *bcr-abl* and *neurofibromin* (Solomon, E. et al. (1991) Science 254:1153-1160), (3) membrane oncogenes such as *neu* and *ret* (Aaronson, A.S.A (1991) Science 254:1153-1161), (4) tumor suppressor genes such as mutant *p53* and mutant *Rb* (*retinoblastoma*) (Weinberg, R.A. (1991) Science 254:1138-1146), and (5) other immortalizing genes such as *Notch dominant negative* (Coffman, C.R. et al. (1993) Cell 23:659-671). Particularly preferred oncogenes include *v-myc* and the *SV40 T antigen*.
- 20 Foreign (heterologous) nucleic acid may be introduced or transfected into multipotent neural stem cells or their progeny. A multipotent neural stem cell or its progeny which harbors foreign DNA is said to be a genetically-engineered cell. The foreign DNA may be introduced using a variety of techniques. In a preferred embodiment, foreign DNA is introduced into multipotent neural stem cells using the technique of retroviral transfection. Recombinant retroviruses harboring the gene(s) of interest are used to introduce marker genes, such as the *E. coli* β -galactosidase (*lacZ*) gene, or oncogenes. The recombinant retroviruses are produced in packaging cell lines to produce culture supernatants having a high titer of virus particles (generally 10^5 to 10^6 pfu/ml). The recombinant viral particles are used to infect cultures
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- of the neural stem cells or their progeny by incubating the cell cultures with medium containing the viral particles and 8 $\mu\text{g}/\text{ml}$ polybrene for three hours. Following retroviral infection, the cells are rinsed
5 and cultured in standard medium. The infected cells are then analyzed for the uptake and expression of the foreign DNA. The cells may be subjected to selective conditions which select for cells that have taken up and expressed a selectable marker gene.
- 10 In another preferred embodiment, the foreign DNA is introduced using the technique of calcium-phosphate-mediated transfection. A calcium-phosphate precipitate containing DNA encoding the gene(s) of interest is prepared using the technique of Wigler et al. (1979)
15 Proc. Natl. Acad. Sci. USA 76:1373-1376. Cultures of the neural stem cells or their progeny are established in tissue culture dishes. Twenty four hours after plating the cells, the calcium phosphate precipitate containing approximately 20 $\mu\text{g}/\text{ml}$ of the foreign DNA is added.
20 The cells are incubated at room temperature for 20 minutes. Tissue culture medium containing 30 μM chloroquine is added and the cells are incubated overnight at 37°C. Following transfection, the cells are analyzed for the uptake and expression of the foreign DNA.
25 The cells may be subjected to selection conditions which select for cells that have taken up and expressed a selectable marker gene.

The following is presented by way of example and is not to be construed as a limitation on the scope of the invention. Further, all references referred to herein
30 are expressly incorporated by reference.

EXAMPLE 1Preparation of Neural Crest Cells

For a given preparation 5-10 timed pregnant female Sprague-Dawley rats (Simonson Laboratories, Gilroy, California) were killed by CO₂ asphyxiation. Embryos were removed and placed into Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, New York) at 4°C for 2-4 hours. Under a dissecting microscope, at room temperature, a block of tissue from a region corresponding to approximately the caudal most 10 somites was dissected from each embryo using an L-shaped electrolytically sharpened tungsten needle. Trunk sections were transferred in HBSS into one well of a 3 well depression slide that had been chilled to 4°C. Trunk sections were treated with collagenase (152 units/mg) (Worthington Biochemical, Freehold, New Jersey) made to a concentration of 0.75 mg/ml in Howard's Ringer's solution (per 1 liter of dH₂O: NaCl 7.2g; CaCl₂ 0.17g; KC1 0.37g) and sterilized, by passage through a 0.22 μm filter prior to use. The collagenase solution was exchanged at least 3 times and with each exchange the trunk sections were vigorously triturated by passage through a pasteur pipet. After incubation at 37°C for 20 minutes in humidified CO₂ atmosphere, the trunk sections were triturated very gently until most of the neural tubes were free and clean of somites and notochords. The collagenase solution was quenched by repeated exchanges with cold complete medium (described below). The neural tubes were plated onto fibronectin-coated (substrate preparation is described below) 60mm tissue culture dishes (Corning, Corning, New York) that had been rinsed with complete medium. After a 30 minute incubation to allow the neural tubes to attach, dishes were flooded with 5 ml of medium. After a 24 hour

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culture period, using an L-shaped electrolytically sharpened tungsten needle and an inverted phase contrast microscope equipped with a 4X objective lens, each neural tube was carefully scraped away from the
5 neural crest cells that had migrated onto the substrate. Crest cells were removed by a 2 minute 37°C treatment with 0.05% Trypsin solution (Gibco). The cells were centrifuged for 4 minutes at 2000 r.p.m. and the pellet was resuspended into 1 ml of fresh complete
10 medium. Typically the cells were plated at a density of 225 cells/100 mm dish.

Substrate Preparation

A. Fibronectin (FN) Substrate

Tissue culture dishes were coated with human plasma
15 fibronectin (New York Blood Center, New York, New York) in the following way. Lyophilized fibronectin was resuspended in sterile distilled water (dH₂O) to a concentration of 10 mg/ml and stored at -80°C until used. The fibronectin stock was diluted to a
20 concentration of 250 mg/ml in Dulbecco's phosphate buffered saline (D-PBS) (Gibco). The fibronectin solution was then applied to tissue culture dishes and immediately withdrawn.

B. Poly-D-Lysine (PDL) and FN Substrate

Sterile poly-D-Lysine (PDL) was dissolved in dH₂O to a
25 concentration of 0.5 mg/ml. The PDL solution was applied to tissue culture plates and immediately withdrawn. The plates were allowed to dry at room temperature, rinsed with 5 ml of dH₂O and allowed to
30 dry again. Fibronectin was then applied, as described above, over the PDL.

EXAMPLE 2

Development of a Defined Medium for
the Growth of Rat Neural Crest Stem Cells

A serum-free, chemically defined basal medium was
5 developed based on the formulations of several existing
defined media. This basal medium consists of L15-CO₂
formulated as described by Hawrot, E. et al. (1979)
Methods in Enzymology 58:574-583 supplemented with
additives described by Bottenstein, J.E. et al. (1979)
10 Proc. Natl. Acad. Sci. USA 76:514-517 and further
supplemented with the additives described by Sieber-
Blum, M. et al. (1985) Exp. Cell Res. 158:267-272. The
final recipe is given here: to L15-CO₂ add, 100 µg/ml
transferrin (Calbiochem, San Diego, California), 5
15 µg/ml insulin (Sigma, St. Louis, MO), 16 µg/ml
putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM
selenious acid (Sigma), 1 mg/ml bovine serum albumin,
crystallized (Gibco), 39 pg/ml dexamethasone (Sigma),
35 ng/ml retinoic acid (Sigma), 5 µg/ml α-d, 1-
20 tocopherol (Sigma), 63 µg/ml p-hydroxybutyrate (Sigma),
25 ng/ml cobalt chloride (Sigma), 1 µg/ml biotin
(Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml
glycerol, 100 ng/ml α-melanocyte stimulating hormone
(Sigma), 10 ng/ml prostaglandin E1 (Sigma), 67.5 ng/ml
25 triiodothyronine (Aldrich Chemical Company, Milwaukee,
Wisconsin), 100 ng/ml epidermal growth factor (Upstate
Biotechnology, Inc., Lake Placid, New York), 4 ng/ml
bFGF (UBI), and 20 ng/ml 2.55 NGF (UBI).

To allow the growth and regeneration of neural crest
30 stem cells in feeder cell-independent cultures, it was
necessary to supplement the basal medium with 10% chick
embryo extract (CEE). This supplemented medium is
termed complete medium.

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CEE is prepared as follows: chicken eggs were incubated for 11 days at 38°C in a humidified atmosphere. Eggs were washed and the embryos were removed, and placed into a petri dish containing sterile Minimal Essential Medium (MEM with Glutamine and Earle's salts) (Gibco) at 4°C. Approximately 10 embryos each were macerated by passage through a 30 ml syringe into a 50 ml test tube (Corning). This typically produced 25 ml of volume. To each 25 ml was added 25 ml of MEM. The tubes were rocked at 4°C for 1 hour. Sterile hyaluronidase (1 mg/25 g of embryo) (Sigma) was added and the mixture was centrifuged for 6 hours at 30,000 g. The supernatant was collected, passed first through a 0.45 µm filter, then through a 0.22 µm filter and stored at -80°C until used.

At the low cell densities necessary for survival and proliferation of individual neural crest cells, either fetal calf serum (FCS, JR Scientific) or CEE was required, in addition to the basal medium, for clone formation. When FCS was used to supplement the medium, it was heat inactivated by treatment at 55°C for 30 minutes. FCS was stored at -20°C and passed through a 0.22 µm filter prior to use.

CEE is preferred as a supplement, as in the presence of FCS, most of the cells derived from the neural crest exhibit a flattened, fibroblastic morphology and expression of LNGFR is extinguished. In the absence of both FCS and CEE, clone formation from neural crest cells was greatly attenuated.

EXAMPLE 3

Isolation and Cloning of
Multipotent Rat Neural Crest Cells

5 A. Identification of Antibody Markers
 Expressed by Neural Crest Cells

In order to identify and isolate rat neural crest cells, it was necessary to identify antibody markers that could be used to recognize these cells. When E10.5 neural tubes were explanted onto a fibronectin 10 (FN) substratum, many of the neural crest cells that emigrated from the neural tubes over the next 24 hours expressed the low-affinity NGF receptor (LNGFR), recognized by monoclonal antibodies 192-Ig and 217c. The outgrowth of neural crest cells from the dorsal 15 side of the explanted neural tube following 24 hours growth in culture is shown in Figure 1, panel A. Figure 1, panel B shows the expression of LNGFR (green fluorescence) and nestin (red fluorescence) in neural crest cells.

20 Neural crest cells were labeled with antibodies as follows: For cell surface antigens, such as LNGFR, it was possible to label the living cells in culture. The cultures were incubated with primary antibody solution for 20 minutes at room temperature. The cultures were 25 washed twice with L15 medium (Gibco) supplemented with 1:1:2, fresh vitamin mix (FVM) (Hawrot, E. et al. (1979), *ibid*), and 1 mg/ml bovine serum albumin (L15 Air). The cultures were then incubated for 20 minutes at room temperature with Phycoerythrin R conjugated 30 secondary antibody (TAGO) at a dilution of 1:200 in L-15 Air. The cultures were then rinsed twice with L-15 Air and placed back in their original medium and examined with a fluorescence microscope. Rabbit anti-LNGFR antiserum (Weskamp, G. et al. (1991) Neuron

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6:649-663) was a kind gift of Gisela Weskamp, University of California, San Francisco and was used at a 1:1000 dilution. Monoclonal anti-NCAM antibody 5A5 (Dodd, J. et al. (1988) Neuron 1:105-116) and 5 monoclonal anti-sulfatide antibody O₄ (Sommer, I. et al. (1981) Dev. Biol. 83:311-327) were obtained as hybridoma cells from the Developmental Studies Hybridoma Bank (Johns Hopkins University, Baltimore, Maryland) and prepared as described by the provider.

10 In order to label cells with antibodies directed against intracellular proteins, it was necessary to fix and permeabilize the cells prior to labeling. For most of the immunocytochemistry, formaldehyde fixation was done. Formaldehyde solution 37% was diluted 1:10 into 15 S-MEM with 1mM HEPES buffer (Gibco). Culture were treated for 10 minutes at room temperature with the 3.7% formaldehyde solution and then rinsed 3 times with D-PBS (Gibco).

20 For some intermediate filament proteins (NF and GFAP) formaldehyde fixation was not possible. Cultures were fixed by treatment with a solution of 95% ethanol and 5% glacial acetic acid at -20°C for 20 minutes.

25 For the staining of cytoplasmic antigens, fixed cells were first treated with a blocking solution comprising D-PBS, 0.1% Tween-20 (Bio-Rad Laboratories, Richmond, California) and 10% heat inactivated normal goat serum (NGS) for 15 minutes at room temperature. Primary antibodies were diluted with a solution of D-PBS, 0.1% Tween-20 and 5% NGS. The fixed cells were incubated 30 overnight at 4°C in primary antibody solution then rinsed twice with DPBS, 0.05% Tween-20. Fluorescent secondary antibodies were diluted with D-PBS, 1% NGS and applied to cells for 1 hour at room temperature. The cells were rinsed twice with D-PBS, 0.05% Tween-20.

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To prevent photobleaching, a solution of 8 mg/ml N-propyl gallate in glycerol was placed over the stained cells prior to fluorescence microscopy.

Mouse monoclonal anti-GFAP, G-A-5 (Debus et al. (1983) 5 Differentiation 25:193-203) was purchased from Sigma and used at a 1:100 dilution. Mouse monoclonal anti-NF200, SMI39 was purchased from Sternberger Monoclonals Inc., Baltimore, Maryland and used at a 1:100 dilution. SMI39 reactivity is equivalent to the O6-53 monoclonal antibody described by Sternberger, L.A. et al. (1983) 10 Proc. Natl. Acad. Sci. USA 80:6126-6130. Purified rabbit antibodies to peripherin (preparation 199-6) was obtained from Dr. Linda Parysek, University of Cincinnati, Ohio and was used at a dilution of 1:500.

15 Flow-cytometric analysis indicated that greater than 70% of the neural crest cells show some LNGFR immunoreactivity (Figure 1, panel D). Approximately 25% of the neural crest cells expressed high levels of LNGFR. In some experiments, neural crest cells expressing high levels of LNGFR were further purified by labeling with 192-Ig (anti-LNGFR) and fluorescence-activated cell sorting (FACS). For single cell analysis, however, it proved more convenient to plate the bulk neural crest cell population at clonal density, and then subsequently identify LNGFR-positive 20 cells by live cell-labeling with 192-Ig.

25

Most or all of the neural crest cells also expressed nestin, an intermediate filament protein found in CNS neuroepithelial cells. An individual neural crest cell 30 co-expressing both nestin and LNGFR is shown in Figure 2, panels A-C. Panel A shows the individual neural crest cell in phase contrast. Panels B and C show this cell following staining with both anti-LNGFR (panel B) and anti-nestin (panel C). Figure 2, panels D-F show

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that the clonal progeny of this nestin⁺, LNGFR⁺ neural crest cell also co-express nestin and LNGFR.

B. Cloning of Multipotent Neural Crest Cells

To define the developmental potential of individual neural crest cells, conditions were established that permit the growth of these cells in clonal culture. Figure 3 provides a flow chart depicting the following cell cloning experiments. In Figure 3, plating medium refers to the complete medium, described above and differentiation medium refers to SCD medium, described below. Using an FCS-free, CEE-containing medium (complete or plating medium), single neural crest cells (Figure 4, panel A, phase contrast and panel B, LNGFR staining) were plated on a FN/PDL substratum and allowed to proliferate and differentiate. After 9-14 days, many of the clones founded by single neural crest cells were large and contained cells with a neuronal morphology (Figure 4, panel C, phase contrast). Quantification indicated that > 60% of the clones contained a mixture of neuronal and non-neuronal cells (see below). These neuronal cells could be labeled by antibodies to pan-neuronal markers such as neurofilament (Figure 4, panel E, anti-NF160 staining) and high-polysialic acid (PSA) NCAM (Figure 4, panel D, anti-NCAM staining), as well as by an antibody to peripherin, an intermediate filament protein that is preferentially expressed by peripheral nervous system (PNS) neurons (Figure 4, panel F). Importantly, these neurons did not express either nestin or LNGFR, indicating that they have lost the two markers that characterize the undifferentiated neural crest cell.

The neuron-containing clones also contained non-neuronal cells. These cells continued to express LNGFR and nestin, in contrast to the neurons, and displayed an elongated morphology characteristic of Schwann

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cells. While immature Schwann cells are known to express both LNGFR and nestin, these markers are insufficient to identify Schwann cells in this system since they are expressed by the neural crest precursor 5 cell as well. Expression of more definitive Schwann cell markers was elicited by transferring the cells into a medium known to enhance Schwann cell differentiation. This medium, called Schwann cell differentiation (SCD) medium, contained both 10% FCS 10 and 5 μ M forskolin, an activator of adenylate cyclase.

Figure 5 shows the expression of a Schwann cell phenotype by neural crest-derived glia. Clones plated initially on FN were allowed to grow for a week in complete medium, then transferred into SCD medium and 15 allowed to grow for another 1-2 weeks prior to fixation and immunocytochemistry. Cells of two morphologies, one elongated and the other flattened can be seen in phase contrast (Panels A and D). To demonstrate concordant expression of three markers, LNGFR, O₄ and 20 GFAP, two different double-labeling experiments were performed. Living cells were surface-labeled with monoclonal anti-LNGFR 192IgG (Panel B) and monoclonal O₄ IgM (Panel C) and postfixed. In parallel, other 25 cells from the same clone were first surface-labeled with O₄ and then fixed with acid-ethanol, permeabilized and stained with anti-GFAP (IgG). Note that LNGFR⁺ cells (Panel B) are O₄⁺ and that most or all of the O₄⁺ cells are also GFAP⁺ (Panels E and F). The quality of 30 the O₄ staining in (Panel E) appears different from that in (Panel C) because a redistribution of the antigen occurs following acid-ethanol fixation. In Panel C, the flattened O₄⁺ cells are more weakly stained for LNGFR (Panel B). Such flattening is indicative of myelination, and is consistent with the

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fact that Schwann cells undergoing myelination down-regulate LNGFR and up-regulate O₄.

Following 5-10 days in SCD medium, most or all of the non-neuronal cells in the clones expressed glial fibrillary acidic protein (GFAP), an intermediate filament specific to glial cells, and sulfatide, a cell-surface glycolipid recognized by the monoclonal antibody O₄. Triple-labeling of such "mature" clones with polyclonal anti-peripherin and monoclonal O₄ and anti-GFAP antibodies revealed that sulfatide and GFAP were not expressed by the peripherin-positive neurons and that these two glial markers were coincident in the non-neuronal cell population (Figure 6). Figure 6 shows a clone from a single founder cell in phase contrast (Panel A) which expresses LNGFR (Panel B). This clone was allowed to proliferate and differentiate in complete medium (containing CEE and lacking serum) and then transferred into SCD medium (containing serum and forskol in). After approximately 10 days, the culture was fixed and triple-labeled with rabbit anti-peripherin (Panels C and D, in green/yellow), anti-GFAP (IgG) (Panel C, in red) and O₄ (IgM) (Panel D, blue). Panels C and D are two separate fields from the same clone.

Although GFAP is expressed by astrocytes and sulfatide is expressed by oligodendrocytes in the CNS, the co-expression of these two markers in the same cell is unique to peripheral glial cells (Jessen, K.R. et al. (1990) Devel. 109:91-103 and Mirsky, R. et al. (1990) Devel. 109:105-116).

Therefore, these data indicate that single neural crest cells expressing nestin and LNGFR are able to give rise to clones of differentiated cells containing both peripheral neurons and glia. Differentiation to the

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neuronal phenotype involves both the loss of LNGFR and nestin expression, and the gain of neuronal markers such as neurofilament, high PSA-NCAM and peripherin. On the other hand, in the glial lineage LNGFR and nestin expression persist, and additional glial markers (GFAP and O₄) are acquired. All clones that produced neurons and glia also produced at least one other cell type that did not express any of the differentiation markers tested; the identity of these cells is unknown. Taken together, these data establish the multipotency of the rat neural crest cell identified and isolated by virtue of co-expression of LNGFR and nestin.

EXAMPLE 4

Self-renewal of Multipotent Neural Crest Cells in vitro

After 10 days in culture in medium supplemented with 10% CEE and on a FN/PDL substrate, all of the neural crest cell clones that contained neurons also contained non-neuronal cells expressing LNGFR and nestin (as described above). In order to determine whether these cells were immature glia, or multipotent neural crest cells that had undergone self-renewal, serial subcloning experiments were performed. Figure 7 provides a flow chart summarizing these serial subcloning experiments. In Figure 7, "plating medium" refers to complete medium containing CEE and lacking FCS and "differentiation medium" refers to SCD medium containing FCS and forskolin.

For serial sub-cloning experiments, clones were harvested and replated as follows. The primary clones were examined microscopically to ensure that there were no impinging colonies and that the whole clone fits

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within the inscribed circle. Using sterile technique throughout the procedure, glass cloning cylinders (3mm id.) were coated on one end with silicone grease (Dow Corning) and placed about the primary clone so that the
5 grease formed a seal through which medium could not pass. The cells were removed from the cylinder by first treating them with 100 ml of 0.05% Trypsin solution (Gibco) for 3 minutes at 37°C in a humidified 5% CO₂ incubator. At room temperature 70 µl of the
10 trypsin solution was removed and replaced with 70 µl of complete medium. The cells were resuspended into the 100 µl volume by vigorous trituration through a pipet tip and the whole volume was diluted into 5 ml of complete medium. The 5 ml was then plated onto 1 or 2
15 60mm dishes which were placed in a humidified 5% CO₂ incubator for 2 hours at which time the medium was exchanged for fresh complete medium. Single founders cells were then identified and allowed to grow into secondary clones as described below.

20 Primary clones founded by LNGFR-positive progenitor cells were allowed to grow for 6 days (Figure 8, Panel A) on a PDL/FN substrate. At this time, clones containing LNGFR-positive cells were identified by live cell surface labeling, and these clones were then
25 removed from their original plates by trypsinization, as described above. The dissociated cells were then replated at clonal density under the same culture conditions as their founder cells. Individual secondary founder cells were identified by labeling live cells with 192-Ig and their positions marked (Figure 8, Panels B and B' show two individual secondary founder cells; Panels C and C' show the clonal progeny of these individual cells at day 17). Both non-neuronal, neurite bearing cells are visible in the clones (Figure
30 8, panels C and C').
35

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A clone derived from secondary founder cells, such as that shown in Figure 8, was transferred into SCD medium to allow the expression of Schwann cell markers. After approximately 10 days, the subclone was fixed, and 5 double-labeled for NF160 and GFAP (Figure 9, Panel A shows the clone in phase contrast; Panel B shows labeling with anti-NF160; Panel C shows labeling with anti-GFAP). The apparent labeling of neurons in panel C is an artifact due to bleed-through into the 10 fluorescein channel of the Texas Red fluorochrome used on the goat anti-rabbit secondary antibody in panel B.

Additionally, following 10 days of secondary culture, living subclones were scored visually for the presence of neurons and glia by double labeling with 192-Ig 15 (anti-LNGFR) and 5A5, a monoclonal antibody to high PSANCAM.

Single neural crest cells isolated from primary clones were able to proliferate and generate clones containing both neurons and non-neuronal cells, probably glia. 20 Quantitative analysis of clones derived from 16 different primary and 151 secondary founders after ten days in plating medium indicated that over 30% of the total secondary founder cells gave rise to clones containing neurons (N), glia (G) and other (O) cells 25 (Table I, N+G+O). Of the remaining 70% of the founder cells, however, almost 50% failed to form clones and died; thus of the clonogenic (i.e., surviving) founders, 54% were of the N+G+O type (Table I). To confirm that these mixed clones indeed contained glia 30 or glial progenitors, they were transferred to SCD medium and allowed to develop for an additional 7 days, then fixed and double-stained for neurofilament and GFAP expression. As was the case for the primary clones, this treatment caused expression of GFAP in a 35 high proportion of non-neuronal cells in the clones

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(Figure 9), confirming the presence of glia. These data indicate that primary neural crest cells are able to give rise at high frequency to progeny cells retaining the multipotency of their progenitors, 5 indicative of self renewal. However, in several cases secondary clones containing only neurons were found (Table I, N only), and many of the secondary clones contained glia and other cells but not neurons (Table I, G+O). This observation suggests that in addition to 10 self-renewal, proliferating neural crest cells may undergo lineage restriction in vitro as well to give rise to glial or neuronal progenitor cells which are characterized by the capacity to divide and self-renew but are restricted to either the neuronal or glial 15 lineage.

EXAMPLE 5

Substrate Composition Influences the Developmental Fate of Multipotent Neural Crest Cells

The foregoing experiments indicate that neural crest 20 cells grown on a PDL/FN substrate generate clones containing both peripheral neurons and glia. When the same cell population is grown at clonal density on a substrate containing FN only, the resulting clones contain glia and "other" cells but never neurons 25 (Figures 10 and 11, Panels D,E,F). Figure 10 provides a flow chart summarizing the following experiments which demonstrate the substrate effect on the fate of mammalian neural crest cells. Figure 11 shows the immunoreactivity of cells stained for various markers.

30 On FN alone, G+O clones are obtained containing non-neuronal cells expressing high levels of LNGFR immunoreactivity, but neither NCAM⁺ nor neurite-bearing

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cells (Figure 11, panels E,F). By contrast on PDL/FN, the clones contain both LNGFR⁺, NCAM⁻ non-neuronal crest cells and LNGFR⁻, NCAM⁺ neurons (Figure 11, panels B,C). Quantification indicated that on FN alone, 70-80% of
5 the clones are of the G+O phenotype and none of the N+G+O phenotype (Figure 12, panel A), whereas on PDL/FN 60% of the clones are of the N+C+O and only 20% are of the GAO phenotype (Figure 12, panel B). These data indicate that the composition of the substrate affects
10 the phenotype of neural crest cells that develop in culture.

To rule out the possibility that the foregoing results could be explained simply by the failure of neurogenic crest cells to adhere and survive on a FN substrate, a
15 different experiment was performed in which all the crest cells were initially cloned on a FN substrate. Figure 13 provides a flow chart summarizing these experiments. These experiments were performed to demonstrate that differences in attachment and/or
20 survival do not account for differences in eventual clone composition. Subsequently, one group of cells was exposed to PDL as an overlay in liquid media (0.05 mg/ml) after 48 hrs, while a sister culture was retained on FN alone as a control (Figure 13). Clones
25 expressing LNGFR were identified by live cell surface labeling at the time of the PDL overlay and the development of only LNGFR⁺ clones was further monitored. After two weeks, the cultures were transferred to SCD medium for an additional 10 days of
30 culture, and their phenotypes then scored as previously described.

By contrast to clones maintained on FN, where no neurons developed, many of the clones exposed to a PDL overlay contained neurons at the end of the culture period (Figure 14, panel A). Moreover, virtually none
35

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of the clones were of the G+O phenotype after the PDL overlay. These data indicate that an overlay of PDL is able to alter the differentiation of neural crest cells even if they are initially plated on an FN substrate.

5 Moreover, they suggest that at least some of the N+G+O clones derived by conversion of founder cells that would have produced G+O clones on FN. However, because of the increased cytotoxicity obtained from the PDL overlay, it was not possible to rule out the

10 possibility that many of the cells that would have produced G+O clones simply died. To address this issue, the PDL overlay was performed on a parallel set of cultures at day 5 rather than at 48 hrs. Under these conditions, virtually all of the LNGFR⁺ clones

15 survived and differentiated. 60% of these clones contained neurons, whereas 35% contained GAO (Figure 14, panel B). By contrast, greater than 90% of the clones maintained on FN developed to a G+O phenotype. Since little or no clone death was obtained under these

20 conditions, and since a majority of the clones contained neurons following the PDL overlay at day 5, these data suggest that PDL converts presumptive G+O clones into N+G+O clones. However the fact that 35% of the clones became G+O following PDL overlay at days,

25 whereas virtually none did so when the overlay was performed at 48 hrs (Fig. 14, compare G+O, hatched bars, in panels A and B), suggests that some clones might become resistant to the effect of PDL between 48 hrs and days.

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EXAMPLE 6

Substrate Influences Latent Developmental
Potential of Neural Crest Cells

To demonstrate more directly that the substrate can alter the developmental fate of neural crest cells, a serial subcloning experiment was performed. Clones were established on FN, and after 5 days the progeny of each clone were subdivided and cloned onto both FN and PDL/FN substrates. Following 10 days of culture in standard medium, the clones were shifted to SCD medium for an additional week to ten days and then fixed, stained and scored for the presence of neurons and Schwann cells. Five of seven primary clones founded on FN gave rise to secondary clones containing neurons when replated onto a PDL/FN substrate at days (Table II). On average, $57 \pm 17\%$ of the secondary clones contained neurons. By contrast, none of the sister secondary clones replated onto FN contained neurons (Table II). These data confirm that the PDL/FN substrate is able to alter the fate of neural crest cell clones initially grown on FN. They also reveal that the "neurogenic potential" of neural crest cells is retained, at least for a period of time, on FN even though overt neuronal differentiation is not observed.

This suggested that FN is non-permissive for overt neuronal differentiation under these culture conditions. In support of this idea, when primary clones established on PDL/FN were replated onto FN, none of the secondary clones contained neurons, whereas 100% (5/5) of the primary clones gave rise to neuron-containing secondary clones when replated onto PDL/FN (Table II). Moreover, on average $93 \pm 7\%$ of the secondary clones derived from each primary clone contained neurons on PDL/ FN, indicating that most or

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all of the clonogenic secondary crest cells retained neurogenic potential under these conditions.

While this experiment indicated that at least some neural crest clones retain neurogenic potential on FM,
5 not all clones exhibited this capacity. This could indicate a heterogeneity in the clonogenic founder cells that grow on FN, or it could indicate a progressive loss of neurogenic potential with time in culture on FM. To address this issue, a second
10 experiment was performed in which primary clones were replated at day 8 rather than at day 5. In this case, a more dramatic difference was observed between primary clones established on FM versus on PDL/FN. Only 1/6 primary FM clones replated at day 8 gave rise to any
15 secondary clones containing neurons on PDL/FN, and in this one case only 17% of the secondary clones contained neurons (Table II). By contrast, 6/6 primary PDL/FN clones gave rise to neuron-containing secondary clones when replated on PDL/FN at day 8, and 52+7% of
20 these secondary clones contained neurons (Table II). These data suggest that neurogenic potential is gradually lost by neural crest cells cultured on FM, but retained to a much greater extent by the same cells grown on PDL/FN. Thus the composition of the substrate influences not only the overt differentiation of the neural crest cells, but also their ability to maintain
25 a latent developmental potential over multiple cell generations.

EXAMPLE 7

Identification of
Neural Crest Stem Cells by Transplantation

Neural crest stem cells are identified by two general
5 criteria: by their antigenic phenotype, and by their
functional properties. These functional properties may
be assessed in culture (*in vitro*), as described above,
or they may be assessed in an animal (*in vivo*). The
above examples described how the self-renewal and
10 differentiation of neural crest stem cells can be
assayed *in vitro*, using clonal cell cultures. However,
these properties may also be determined by
transplanting neural crest cells into a suitable animal
host. Such an assay requires a means of delivering the
15 cells and of identifying the transplanted cells and
their progeny so as to distinguish them from cells of
the host animal. Using standard techniques, it is
possible to deliver neural crest cells to a developing
mammalian or avian embryo or to any tissue or
20 compartment of the adult animal (e.g., brain,
peritoneal cavity, etc.).

For example, neural crest cell cultures are prepared as
described earlier. After a suitable period in primary
or secondary culture, neural crest cells are identified
25 by live cell-labeling with antibodies to LNGFR, and
removed from the plate using trypsin and a cloning
cylinder, as described in previous examples. The cells
are diluted into serum-containing medium to inhibit the
trypsin, centrifuged and resuspended to a concentration
30 of 10^6 - 10^7 cells per milliliter. The cells are
maintained in a viable state prior to injection by
applying them in small drops (ca. 10 μ l each) to a 35
mm petri dish, and evaporation is prevented by
overlaying the droplets with light mineral oil. The

cells are kept cold by keeping the petri dishes on ice. For injections into mouse embryos, pregnant mothers at embryonic day 8.5 - 9.0 are anaesthetized and their uterus exposed by an incision into the abdomen. Neural
5 crest cells are drawn into a sharpened glass micropipette (with a sealed tip and hole in the side to prevent clogging during penetration of tissues) by gentle suction. The pipette is inserted into the lower third of the deciduum and a volume of approximately 0.5
10 μl is expelled containing approximately 1000 cells. The micropipette is withdrawn and the incision is sutured shut. After an additional 3-4 days, the mother is sacrificed, and individual embryos are removed,
15 fixed and analyzed for the presence and phenotype of cells derived from the injected neural crest cells.

To identify the progeny of the injected cells, it is necessary to have a means of distinguishing them from surrounding cells of the host embryo. This may be done as follows: rat neural crest cells are injected into
20 a mouse embryo (following suitable immunosuppression of the mother or using a genetically immunodeficient strain such as the SCID strain of mice), the injected cells are identified by endogenous markers such as Thy1 or major histocompatibility complex (MHC) antigens
25 using monoclonal antibodies specific for the rat Thy1 or MHC antigens. Alternatively, an exogenous genetic marker is introduced into the cells prior to their transplantation as a means of providing a marker on or in the injected cells. This is as follows: neural
30 crest cells in culture are incubated with a suspension of replication-defective, helper-free retrovirus particles harboring the lacZ gene, at a titer of 10^5 - 10^6 pfu/ml in the presence of 8 $\mu\text{l}/\text{ml}$ polybrene for four hours. The cells are then washed several times
35 with fresh medium and prepared for injection as described above. The harvested embryos are then

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assayed for expression of β -galactosidase by whole mount staining according to standard procedures. The blue cells (indicating expression of the lacZ gene) will correspond to the progeny of the injected neural 5 crest cells. This procedure can be applied to any tissue or any stage of development in any animal suitable for transplantation studies. Following whole-mount staining, embryos bearing positive cells are embedded in freezing medium and sectioned at 10-20 μm 10 on a cryostat. Sections containing blue cells are selected, and then counterstained for markers of mature neurons and glia using specific antibodies, according to standard techniques, and immunoperoxidase or alkalinephosphatase histochemistry. The identification 15 of lacZ+ (blue) cells expressing neuronal or glial markers indicates that the progeny of the injected neural crest cells have differentiated appropriately. Thus, this technique provides a means of identifying mammalian neural crest stem cells through 20 transplantation studies to reveal the function of said stem cells.

EXAMPLE 8

Genetic-Engineering of
Neural Crest Stem Cells (NCSCs)

25 A. Retroviral infection of NCSCs

In this method, NCSCs are infected with a replication-incompetent, recombinant retrovirus harboring the foreign gene of interest. This foreign gene is under the control of the long terminal repeats (LTRs) of the 30 retrovirus, in this case a Moloney Murine Leukemia Virus (MoMuLv) (Cepko et al. (1984) Cell 37:1053-1062). Alternatively, the foreign gene is under the control of a distinct promoter-enhancer contained within the

recombinant portion of the virus (i.e., CMV or RSV LTR). In this particular example, the *E. coli* β -galactosidase gene was used, because it provides a blue histochemical reaction product that can easily be used
5 to identify the genetically-engineered cells, and thereby determine the transformation efficiency.

Rat NCSC cultures were established as described above. Twenty-four hours after replating, the cells were exposed to a suspension of β -galactosidase-containing
10 retrovirus (Turner et al. (1987) Nature 328:131-136) with a titer of approximately 10^5 - 10^6 pfu/ml in the presence of 8 μ g/ml polybrene. Following a 3 hr exposure to the viral suspension, the cultures were rinsed and transferred into standard medium. After
15 three days of growth in this medium, the transformed cells were visualized using the X-gal histochemical reaction (Sanes et al. (1986) EMBO J. 5:3133-3142) Fig. 15, Panel A shows the NCSC culture three days after infection with the lacZ containing retrovirus, after fixation and staining using the X-gal reaction. β -galactosidase-expressing cells are indicated by the solid arrows. Non-expressing cells in the same
20 microscopic field are visualized by phase contrast microscopy (B), and are indicated by open arrows. The blue, β -galactosidase⁺ cells represented approximately 5-10% of the total cells in the culture as visualized
25 by phase-contrast microscopy (Fig. 15, Panel B).

B. Calcium-Phosphate-Mediated Transfection of NCSCs
In this method, NCSCs are transfected with an
30 expression plasmid using the calcium phosphate method (Wigler et al. (1979) Proc. Natl. Acad. Sci. USA 76:1373-1376). As in the previous example, the β -galactosidase gene was used to facilitate visualization of the transfected cells.

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In this case, the vector pRSVlacZ was used, in which the β -galactosidase gene (lacZ) is under the control of the Rous Sarcoma Virus (RSV) LTR, and the SV40 intron and poly A-addition site are provided at the 3' end of 5 the gene (Johnson et al. (1992) Proc. Natl. Acad. Sci. USA 89:3596-3600).

NCSCs were established in 35 mm tissue culture dishes. 24 hr after plating, a calcium phosphate precipitate containing approximately 20 μ g/ml of pRSVlacZ was 10 prepared. 123 μ l of this precipitate was added to each dish, and incubated at room temperature for 20 minutes. Two ml of standard medium containing 30 μ M chloroquine was then added to each dish and incubation was continued overnight at 37°C. The next day, the medium 15 was replaced and incubation continued for a further two days. The cultures were then fixed and assayed for β -galactosidase expression by the standard X-gal reaction. Approximately 10% of the NCSCs expressed the lacZ reaction product.

20 C. Immortalization of NCSCs

NCSC cultures are established as described above. The cultures are exposed, in the presence of 8 μ g/ml polybrene, to a suspension of retrovirus harboring an oncogene preferably selected from the immortalizing 25 oncogenes identified herein. These retroviruses contain, in addition to the oncogene sequences, a gene encoding a selectable marker, such as hisD, driven by the SV40 early promoter-enhancer (Stockschlaeder, M.A.R. et al. (1991) Human Gene Therapy 2:33). Cells 30 which have taken up the hisD gene are selected for by growth in the presence of L-histidinol at a concentration of 4 mM. Alternatively, selection can be based upon growth in the presence of neomycin (500 μ g/ml). NCSCs are infected with the above 35 retroviruses which are concentrated to a titer of

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greater than 10^6 pfu/ml by centrifugation. The virus is applied to the cells in two sequential incubations of 4-8 hours each in the presence of 8 μ g/ml polybrene.

Following infection, the cells are grown in the presence of 4 mM L-histinol or 500 μ g/ml neomycin (G418) for 5-10 days. Cells which survive the selection process are screened for expression of LNGFR by live-cell labeling using the monoclonal antibody 192 Ig as described above. Colonies containing a homogeneous population of LNGFR+ cells are cloned using a cloning cylinder and mild trypsinization, and transferred into duplicate FN/pDL-coated 96-well plates. After a short period of growth, one of the plates is directly frozen (Ramirez-Solis, R. et al. (1992) Meth. Enzymol., in press). The cells in the other plate are replated onto several replicate 96-well plates, one of which is maintained for carrying the lines. The cells on the other plates are fixed and analyzed for the expression of antigenic markers. Successful immortalization is indicated by (1) the cells homogeneously maintain an antigenic phenotype characterized by LNGFR+, nestin+, lin- (where "lin" refers to lineage markers characteristic of differentiated neuronal or glial crest derivatives, including neurofilament, peripherin, hi PSA-NCAM, GFAP, O4 and P₀); and (2) the cell population is phenotypically stable over several weeks of passage (as defined by lack of differentiation to morphologically- and antigenically-recognizable neurons and/or glia). The ability of the lines to differentiate is tested by transferring them to conditions that promote differentiation (omission of CEE in the case of neurons and addition of serum and 5 μ M forskolin for Schwann cells). Maintenance of the ability to differentiate is a desirable, although not necessary, property of the constitutively-immortalized cells.

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EXAMPLE 9Generation of Monoclonal Antibody to Mouse LNGFR

Mouse monoclonal antibodies specific to LNGFR from primates (Loy et al. (1990), J. Neurosci. Res. 27:657-664) and rat (Chandler et al. (1984) J. Biol. Chem. 259:6882-6889) have been produced. No monoclonal antibodies to mouse LNGFR have been described. We have produced rat monoclonal antibodies to mouse LNGFR. These antibodies recognize epitopes present on the surface of living cells such as Schwann cells, making them suitable for use in immunologic isolation of multipotent neural stem cells (such as neural crest stem cells) and their differentiated derivatives (as well as neural progenitor cells from the CNS) from murine species. The isolation of such cells from mice is particularly desirable, as that species is the experimental organism of choice for genetic and immunological studies or human disease.

To generate monoclonal antibodies to mouse LNGFR, a genomic DNA fragment encoding the extracellular domain (ligand binding domain) of that protein was expressed in *E. coli*, as a fusion protein with glutathione-S-transferase (Lassar et al. (1989) Cell 58:823-831). Briefly, a probe for the extracellular domain based on either of the known DNA sequences for rat and human LNGFR is used to screen a mouse genomic library. A cloned insert from a positively hybridizing clone is excised and recombined with DNA encoding glutathione with appropriate expression regulation sequences and transfected into *E. coli*. The fusion protein was affinity-purified on a glutathione-Sepharose column, and injected into rats. Sera obtained from tail bleeds of the rats were screened by surface-labeling of live Schwann cells isolated from mouse sciatic nerve by

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standard procedures (Brockea et al. (1979) In Vitro 15:773-778. Surface labeling was with labelled goat anti-rat antibody. Following a boost, fusions were carried out between the rat spleen cells and mouse myeloma cells. Supernatants from the resulting hybridoma cultures were screened using the live Schwann cell assay. Positive clones were re-tested on NIH 3T3 fibroblasts, a mouse cell line that does not express LNGFR, and were found to be negative. The use of this live cell assay ensures that all antibodies selected are able to recognize LNGFR on the surface of living cells. Moreover the assay is rapid, simple and more efficient than other assays such as ELISA, which require large quantities of purified antigen.

15 Approximately 17 independent positive hybridoma lines were identified and subcloned. An example of the results obtained with the supernatant from one such line 19 shown in Figure 16. A culture of mouse sciatic nerve Schwann cells was labeled with one of the rat anti-mouse LNGFR monoclonal antibodies and counterstained with DAPI to reveal the nuclei of 611 cells. The left panel (A) shows that most of the cells are labeled on their surface with the anti-LNGFR antibody (red staining; solid arrows), the right panel (B) reveals all the cell nuclei on the plate, and shows a few cells not labeled by the anti-LNGFR antibody (blue staining; open arrows; compare to left panel). These unlabeled cells most likely represent contaminating fibroblasts which are known not to express LNGFR. These cells provide an internal control which demonstrates the specificity of the labeling obtained with the anti-LNGFR antibody.

WHAT IS CLAIMED IS:

1. An isolated cellular composition comprising at least one mammalian multipotent neural stem cell capable of self-renewal in a feeder cell-independent culture medium and capable of differentiation to at least neuronal or glial progenitor cells.
5
2. A cellular composition according to Claim 1, wherein said stem cell is capable of differentiating to at least peripheral nervous system neuronal or glial progenitor cells and is further characterized by the presence of a low-affinity nerve growth factor receptor⁺.
10
3. A cellular composition according to Claim 2, wherein said stem cell is further characterized by being nestin⁺.
15
4. A cellular composition according to Claim 3, wherein said cells are further characterized as lacking one or more markers characteristic of mature neuronal or glial cells of the peripheral nervous system.
20
5. The cellular composition of Claim 4 wherein said markers are selected from the group consisting of sulfatide, glial fibrillary acidic protein, peripherin and a neurofilament.
25
6. An isolated cellular composition comprising a mammalian peripheral nervous system glial progenitor cell.
7. A method for obtaining a cellular composition comprising mammalian neural crest stem cells, said method comprising:

a) contacting an embryonic neural tube with a first culture medium and a first substrate to produce a suspension comprising a population of neural crest cells that have emigrated from said embryonic neural
5 tube and

b) contacting at least a portion of said suspension comprising one or more cells of said population with a second culture medium and a second substrate which permit self-regeneration and
10 differentiation of one or more neural crest stem cells in said population.

8. A method according to Claim 7 further comprising the step:

c) identifying at least one stem cell by its ability to self-renew and differentiate in feeder cell-independent culture.
15

9. The method of Claim 8 further comprising prior to or after step b) the step of identifying at least one neural crest stem cell which is characterized by the presence of low-affinity nerve growth factor receptor
20 on the surface of said neural crest stem cell.

10. The method of Claim 7, wherein said differentiation of said stem cells is to at least peripheral nervous system neuronal or glial progenitor
25 cells.

11. The method of Claim 7, wherein said first and second substrates comprise fibronectin.

12. The method of Claim 7, wherein said first and second substrates comprise poly-D-lysine in combination
30 with fibronectin.

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13. The method of Claim 7, wherein said second culture medium further comprises a factor permissive for peripheral nervous system neuronal cell differentiation.

5 14. The method of Claim 13, wherein said factor permissive for peripheral nervous system neuronal cell differentiation is a substrate comprising poly-D-lysine in combination with fibronectin.

10 15. The method of Claim 7, wherein said second culture medium further comprises a factor permissive for peripheral nervous system glial cell differentiation.

16. The method of Claim 15, wherein said factor permissive for peripheral nervous system glial cell differentiation is a substrate comprising fibronectin.

15 17. The method of Claim 15, wherein said factor permissive for peripheral nervous system glial cell differentiation is forskolin.

18. An isolated cellular composition comprising a mammalian peripheral nervous system neuronal progenitor cell made according to the method of Claim 13.

20 19. An isolated cellular composition comprising a mammalian peripheral nervous system neuronal cell made according to the method of Claim 13.

25 20. An isolated cellular composition according to Claim 19, wherein said neuronal cells are characterized by being nestin⁺, low-affinity nerve growth factor receptor⁺, peripherin⁺, and neurofilament⁺.

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21. An isolated cellular composition comprising mammalian peripheral nervous system glial progenitor cells made according to the method of Claim 15.

5 22. An isolated cellular composition comprising mammalian peripheral nervous system glial cells made according to the method in Claim 15.

10 23. An isolated cellular composition according to Claim 22, wherein said glial cells are characterized as being low-affinity nerve growth factor receptor⁺, nestin⁺, glial fibrillary acidic protein⁺, and sulfatide⁺.

15 24. A method for obtaining a cellular composition from a mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor of said progenitor cells, said method comprising:

- 20 a) preparing a suspension comprising a population of cells from said mammalian tissue,
b) contacting said cell suspension with a first culture medium and a first substrate which permit self-renewal of one or more glial or neuronal progenitor cells or multipotent stem cell precursors, and
c) identifying said glial or neuronal progenitor cells or multipotent stem cell precursors, if present, by their ability to self-renew and differentiate in feeder-cell independent culture.

25 25. A method according to Claim 24 further comprising the step:

- 30 c) contacting said glial or neuronal progenitor cells or multipotent stem cell precursors of said progenitor cells with a second culture medium and a second substrate which permit differentiation of one or

more of said glial or neuronal progenitor cells or multipotent stem cell precursors.

26. A method according to Claim 25 further comprising prior to step b):

5 e) isolating one or more cells expressing a neural cell-specific surface marker.

27. A method according to Claim 26 wherein said neural cell-specific surface marker comprises the low affinity nerve growth factor receptor.

10 28. A method according to Claim 24 wherein said cells have at least one property characteristic of a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof.

15 29. An isolated cellular composition comprising a glial or neuronal progenitor cell or multipotent stem cell precursor thereof made according to the method of Claim 24.

20 30. An isolated cellular composition comprising a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof made according to the method of Claim 28.

25 31. A method for obtaining a cellular composition from a mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor of said progenitor cells, said method comprising:

a) preparing a suspension comprising a population of cells from said mammalian tissue and
30 b) transplanting at least of cell of said population into a suitable host animal and

c) identifying a glial or neuronal progenitor cell or multipotent stem cell precursor, if present, by the ability of said cell to self-renew and differentiate into at least the glial and neuronal lineages *in vivo*.
5

32. A method according to Claim 31 further comprising prior to step b):

d) isolating one or more expressing a neural cell-specific surface marker.

10 33. A method according to Claim 32 wherein said neural cell-specific surface marker comprises the low-affinity nerve growth factor receptor.

15 34. A method for obtaining a cellular composition from a mammalian tissue comprising one or more cells having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor of said progenitor cells, said method comprising:

20 a) preparing a suspension comprising cells from said mammalian tissue,

b) contacting said suspension with an antibody capable of forming a first complex with a neural cell-specific surface marker on said glial or neuronal progenitor cells or multipotent stem cell precursor, and
25

c) isolating said first complex, if formed, from said suspension to obtain said cellular composition.

30 35. A method according to Claim 34 wherein said neural cell-specific surface marker comprises the low-affinity nerve growth factor receptor.

36. A method according to Claim 34 wherein said isolating comprises forming a second complex by

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contacting said first complex with a reagent comprising a first member of a binding pair wherein the second member of said binding pair comprises said antibody or a conjugate of said antibody.

5 37. A method according to Claim 36 wherein said reagent contains a fluorescent label and said isolating further comprises separating said second complex, if formed, by fluorescence-activated cell sorting.

10 38. A method according to Claim 34 wherein said one or more cells have at least one property characteristic of peripheral nervous system glial or neuronal progenitor cells or multipotent stem cell precursors of said peripheral nervous system progenitor cells.

15 39. A method comprising introducing one or more cells comprising neuronal or glial progenitor cells or multipotent stem cell precursors thereof into a host animal.

40. A method according to Claim 39 wherein said cells are made according to the method of Claims 24 or 34.

20 41. A method according to Claim 39 wherein said cells comprise peripheral nervous system glial or neuronal progenitor cells or multipotent neural crest stem cell precursors of said peripheral nervous system progenitor cells.

25 42. A method according to Claim 41 wherein said cells are made according to the method of any one of Claims 7, 13, 15 and 29.

43. A method according to Claim 39 or 41 wherein said host animal has a disorder of the nervous system.

44. A method for immortalizing a cell line having at least one property characteristic of a glial or neuronal progenitor cell or multipotent stem cell precursor thereof comprising transfecting said cell 5 with a vector comprising an immortalizing gene.
45. A method according to Claim 44 wherein said cell line has at least one property characteristic of a peripheral nervous system glial or neuronal progenitor cell or multipotent stem cell precursor thereof.
- 10 46. An immortalized cell line made according to the method of Claim 44 or 45.
47. A cellular composition comprising at least one genetically-engineered multipotent neural stem cell.
- 15 48. The cellular composition of Claim 47 wherein said stem cell comprises a neural crest stem cell.
49. A cellular composition comprising at least one differentiated progeny of a genetically-engineered multipotent neural stem cell.
- 20 50. The cellular composition of Claim 49 wherein said stem cell comprises a neural crest stem cell.
51. A cellular composition according to Claim 50, wherein said progeny of said differentiated progeny comprises a peripheral nervous system neuronal progenitor cell.
- 25 52. A cellular composition according to Claim 50, wherein said progeny of said differentiated progeny comprises a peripheral nervous system glial progenitor cell.

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53. A method for generating a genetically-engineered multipotent neural stem cell, said method comprising contacting a multipotent neural stem cell with foreign nucleic acid under conditions permissive for the uptake 5 of said foreign nucleic acid into said stem cell.

54. A method according to Claim 53, wherein said conditions permissive for the uptake of said foreign nucleic acid comprise calcium phosphate-mediated transfection.

10 55. A method according to Claim 53, wherein said conditions permissive for the uptake of said foreign nucleic acid comprise retroviral infection.

56. A monoclonal antibody capable of binding to mouse low-affinity nerve growth factor receptor.

15 57. A method for detecting the presence of an antibody capable of binding a surface marker characteristic of a neural cell comprising:

immunizing an animal with said surface marker;
contacting a live neural cell displaying said 20 surface marker with antibody-containing sera from said immunized animal or antibody produced by a hybridoma formed from a cell obtained from said immunized animal, and

25 detecting whether antibody specific for said surface marker binds to said neural cell.

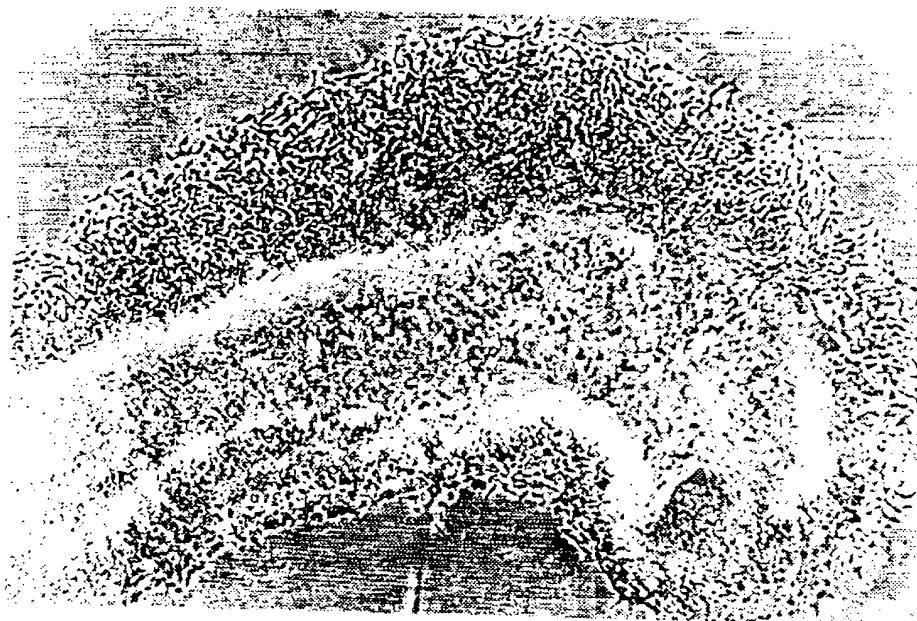


FIG._1A

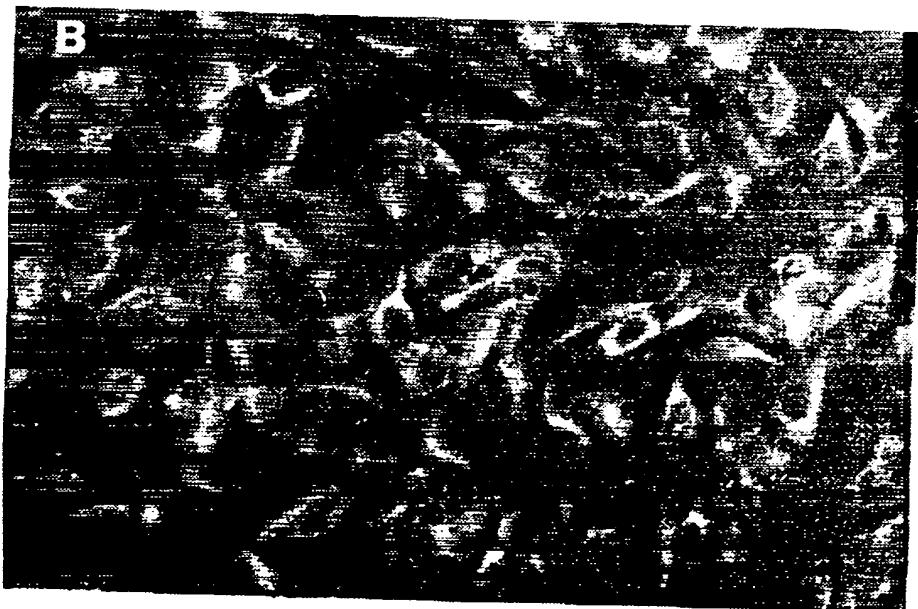


FIG._1B

SUBSTITUTE SHEET (RULE 26)

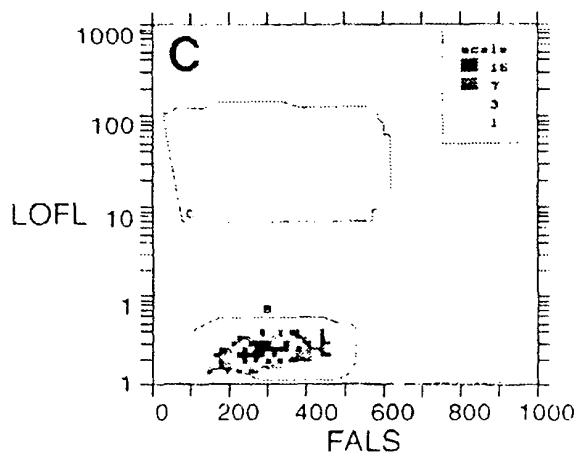


FIG._1C

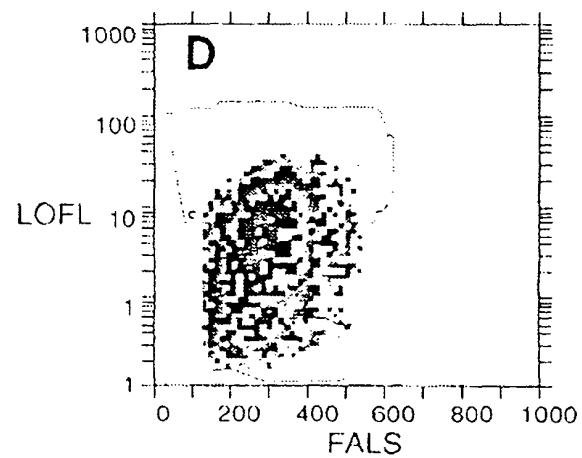
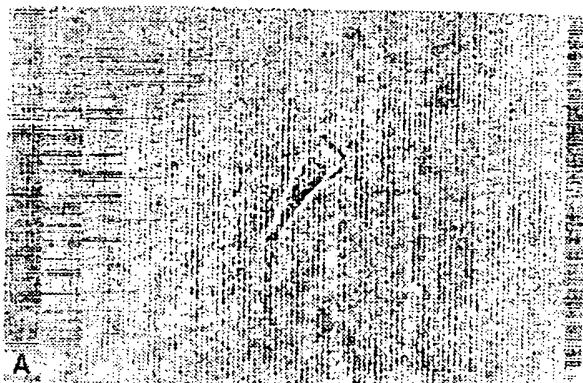
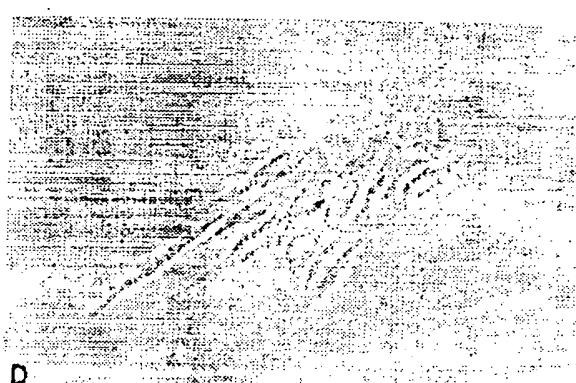
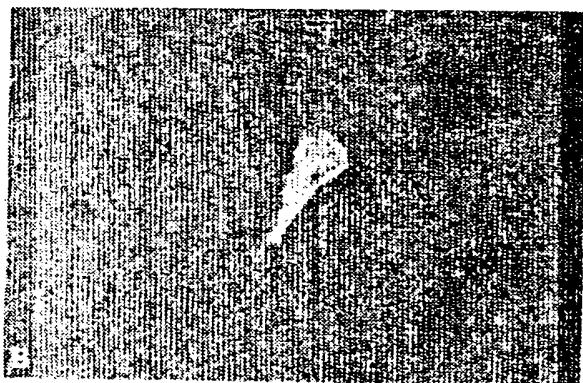
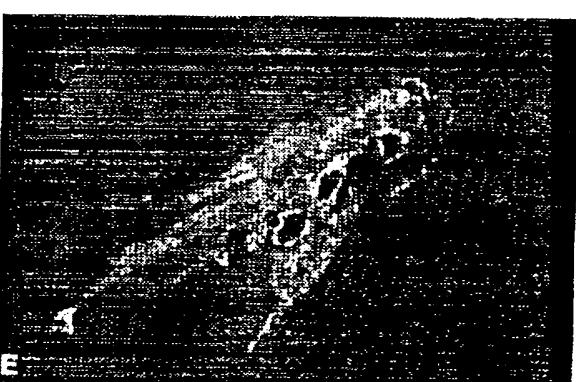
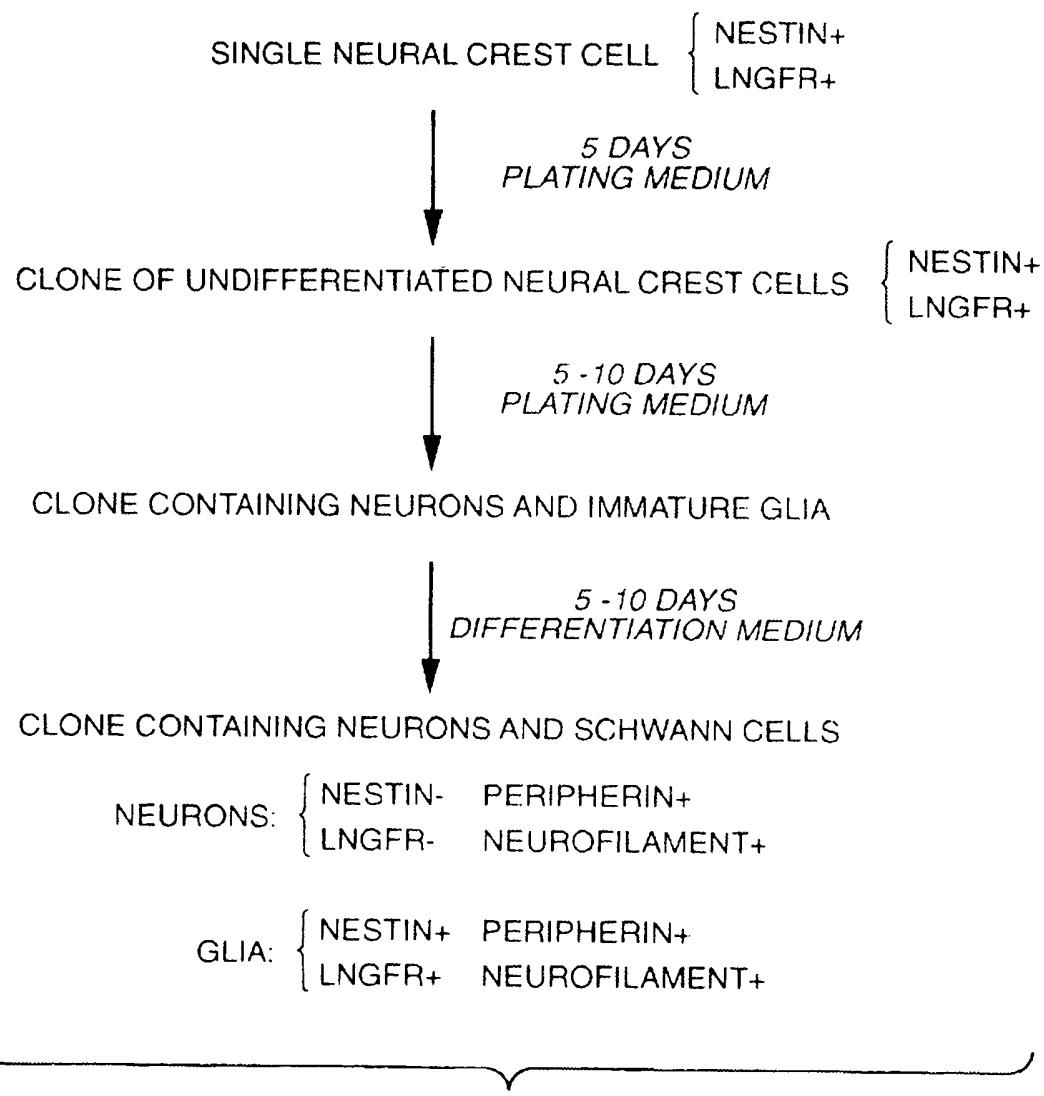
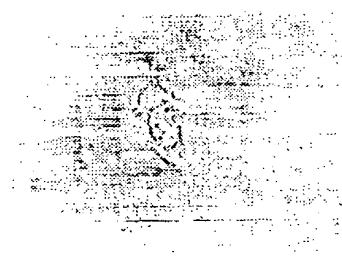
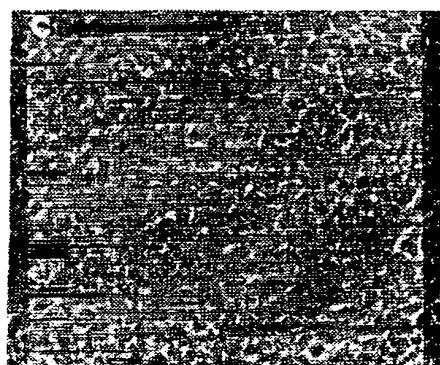
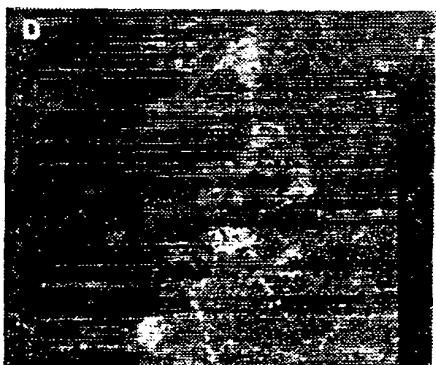
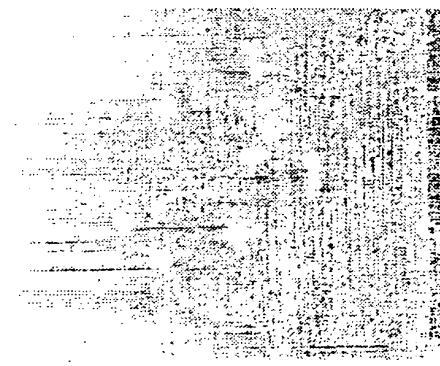


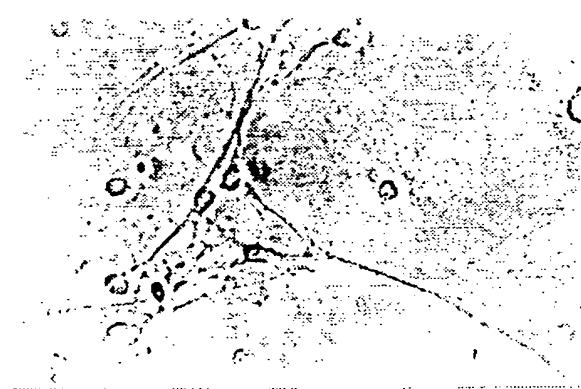
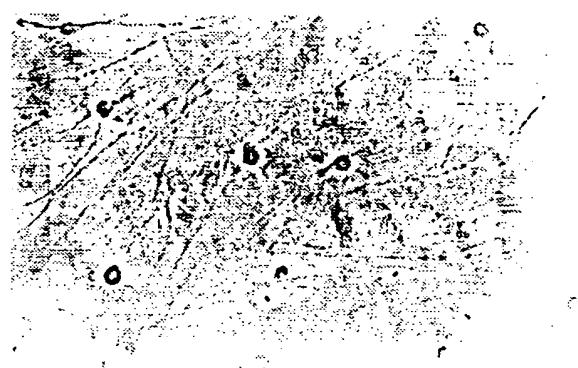
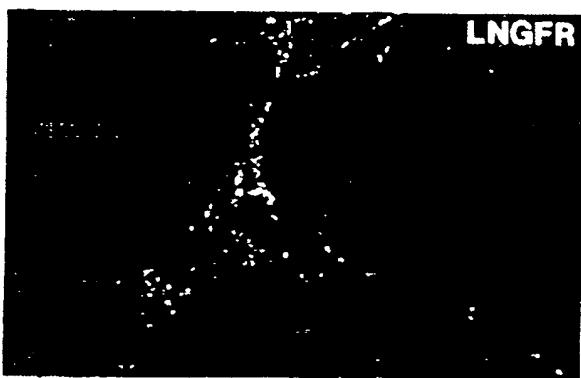
FIG._1D

SUBSTITUTE SHEET (RULE 26)

**FIG._2A****FIG._2D****FIG._2B****FIG._2E****FIG._2C****FIG._2F****SUBSTITUTE SHEET (RULE 26)**

MULTIPOTENCY OF MAMMALIAN NEURAL CREST CELLS**FIG._3****SUBSTITUTE SHEET (RULE 26)**

A**FIG._4A****B****FIG._4B****C****FIG._4C****D****FIG._4D****E****FIG._4E****F****FIG._4F****SUBSTITUTE SHEET (RULE 26)**

**FIG._5A****FIG._5D****FIG._5B****FIG._5E****FIG._5C****FIG._5F**

SUBSTITUTE SHEET (RULE 26)

A

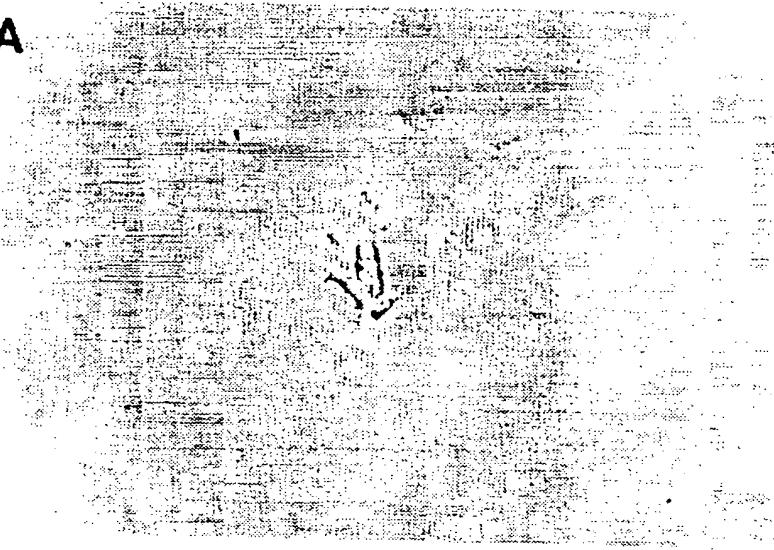


FIG._6A

B



FIG._6B

SUBSTITUTE SHEET (RULE 26)

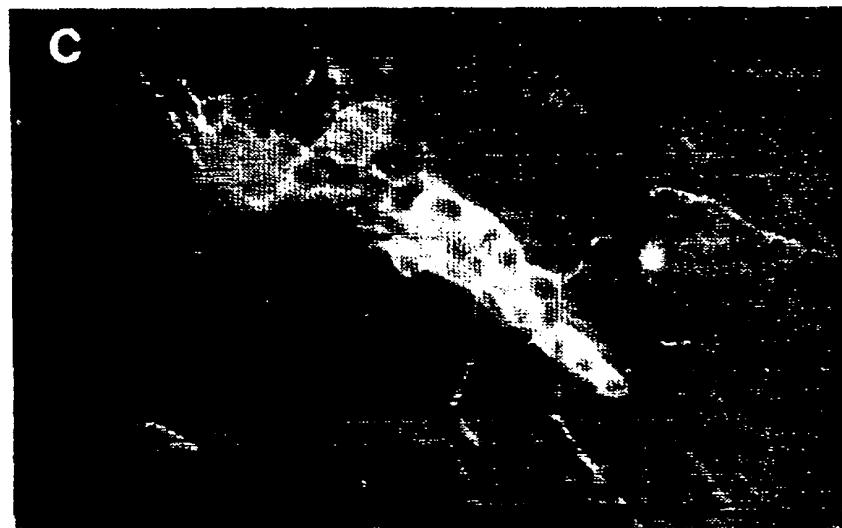


FIG._6C



FIG._6D

SUBSTITUTE SHEET (RULE 26)

SELF-RENEWAL OF MAMMALIAN NEURAL CREST CELLS

SINGLE NEURAL CREST FOUNDER CELL

5 - 7 DAYS
PLATING MEDIUM

CLONE OF UNDIFFERENTIATED NEURAL CREST CELLS

RECLONE AND IDENTIFY SECONDARY FOUNDER CELLS

10 DAYS
DIFFERENTIATION MEDIUM

SCORE CLONES AS MIXED, GLIAL OR OTHER

10 DAYS
DIFFERENTIATION MEDIUM

FIX AND STAIN FOR ANTIGENIC MARKERS

FIG._7

10 / 18

1° CLONE (DAY 7)

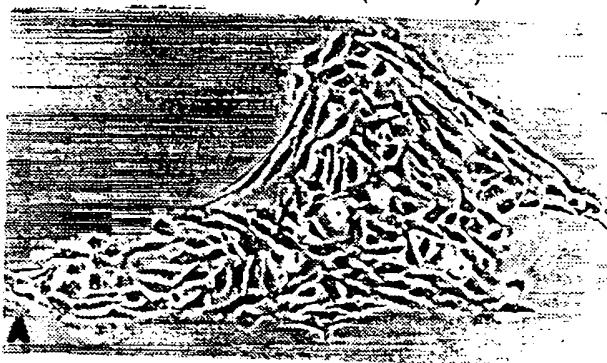
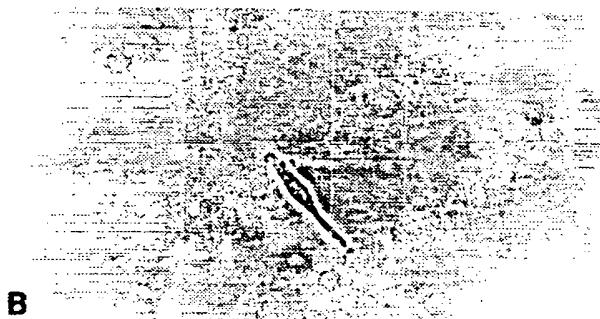


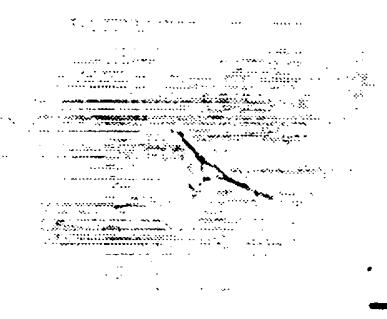
FIG._8A

2° FOUNDERS



B

2° FOUNDERS

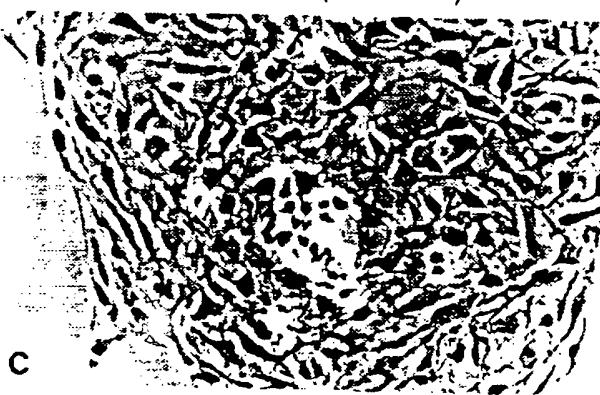


B'

FIG._8B

FIG._8B'

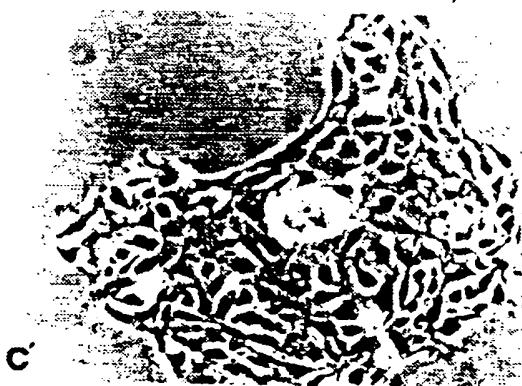
2° CLONES (DAY 17)



C

FIG._8C

2° CLONES (DAY 17)



C'

FIG._8C'

SUBSTITUTE SHEET (RULE 26)

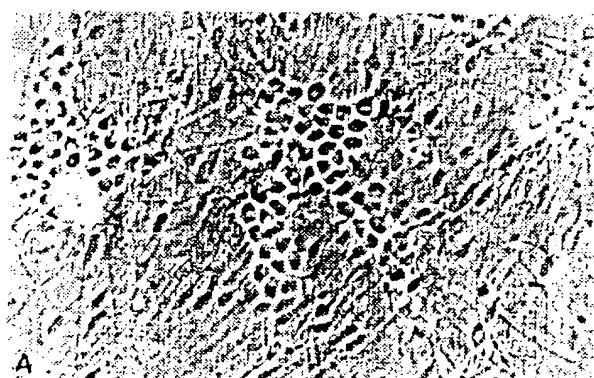


FIG._9A

NF160



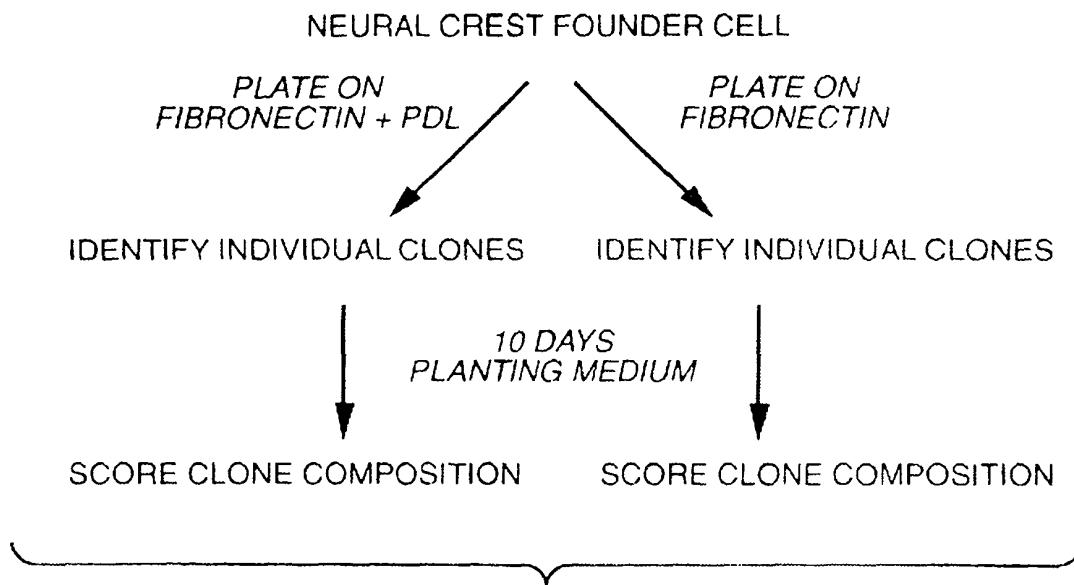
FIG._9B

GFAP

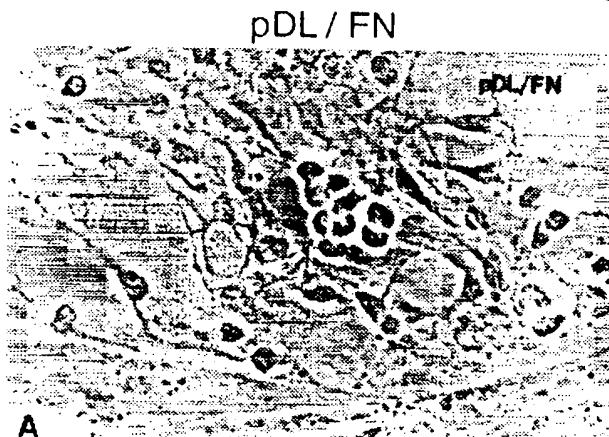


FIG._9C

SUBSTITUTE SHEET (RULE 26)

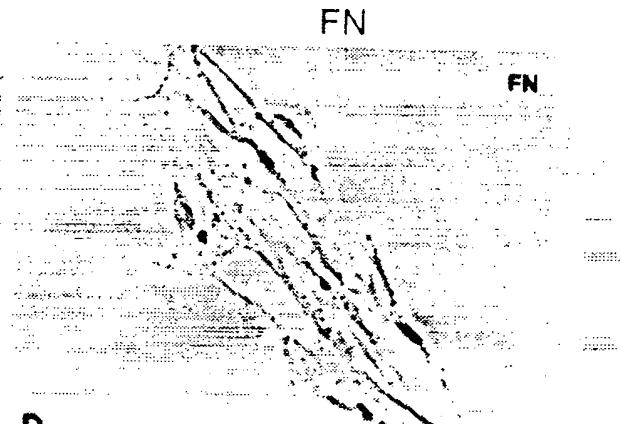
SUBSTRATE EFFECT ON FATE OF MAMMALIAN NEURAL CREST CELLS**FIG._10****SUBSTITUTE SHEET (RULE 26)**

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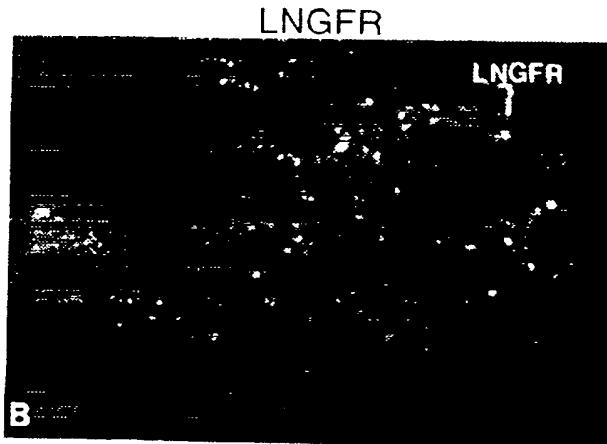
A

FIG._ 11A



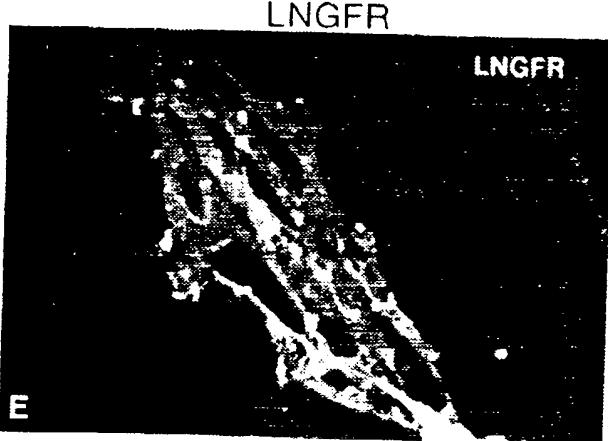
D

FIG._ 11D



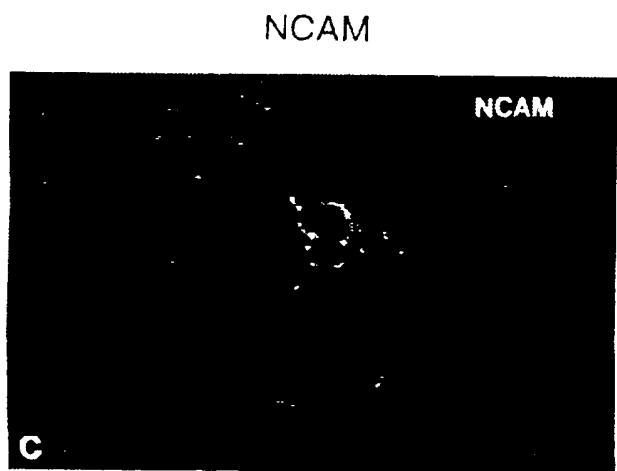
B

FIG._ 11B



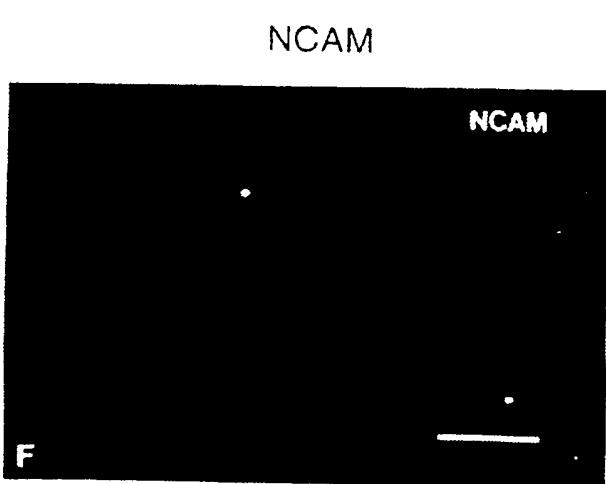
E

FIG._ 11E



C

FIG._ 11C



F

FIG._ 11F

SUBSTITUTE SHEET (RULE 26)

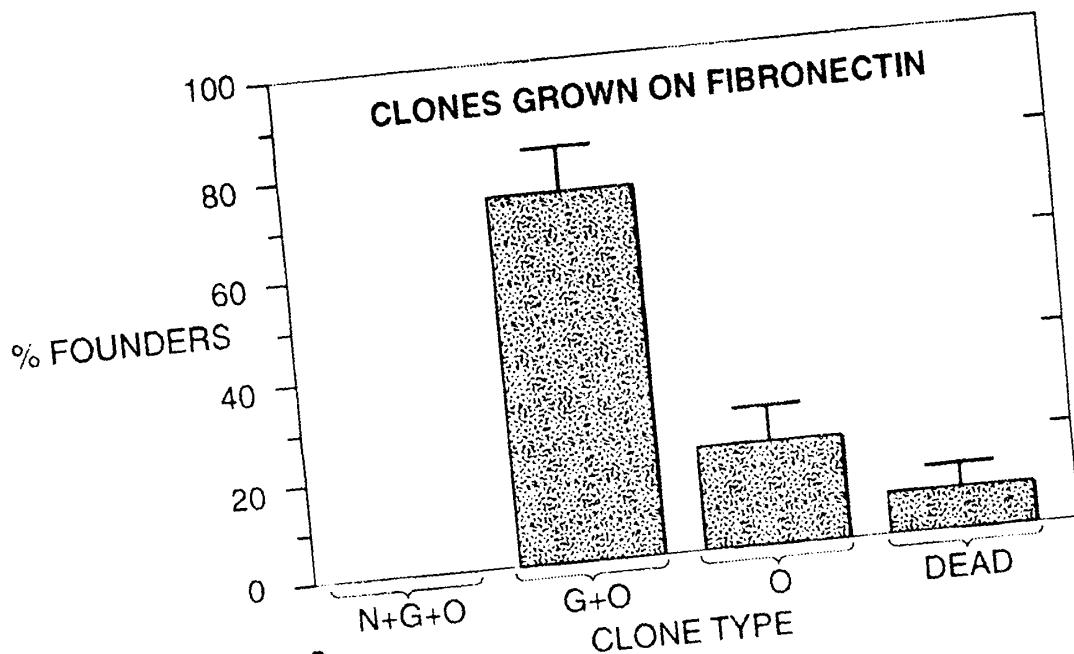


FIG.- 12A

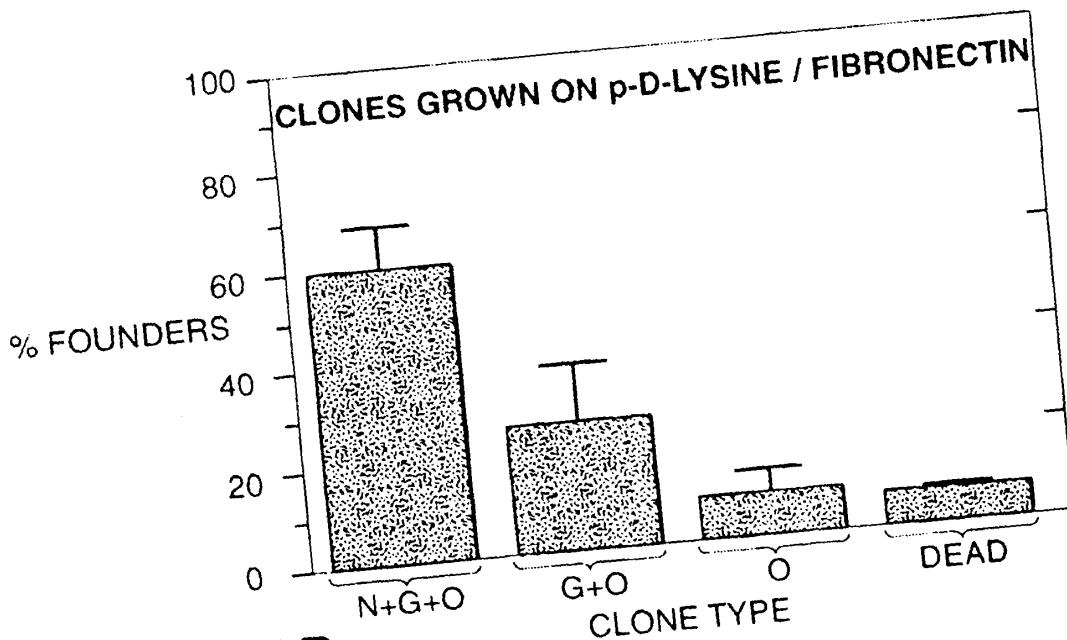
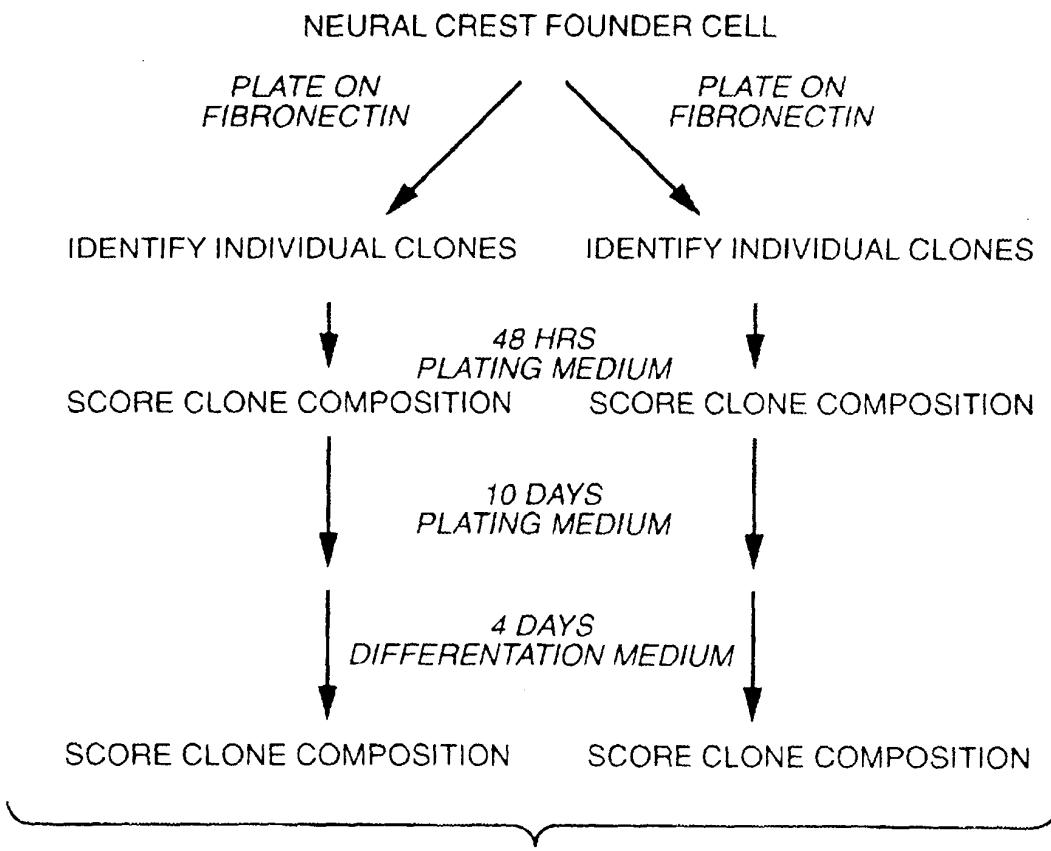


FIG.- 12B

SUBSTITUTE SHEET (RULE 26)

INSTRUCTIVE EFFECT OF SUBSTRATE ON CREST CELL FATE**FIG._13****SUBSTITUTE SHEET (RULE 26)**

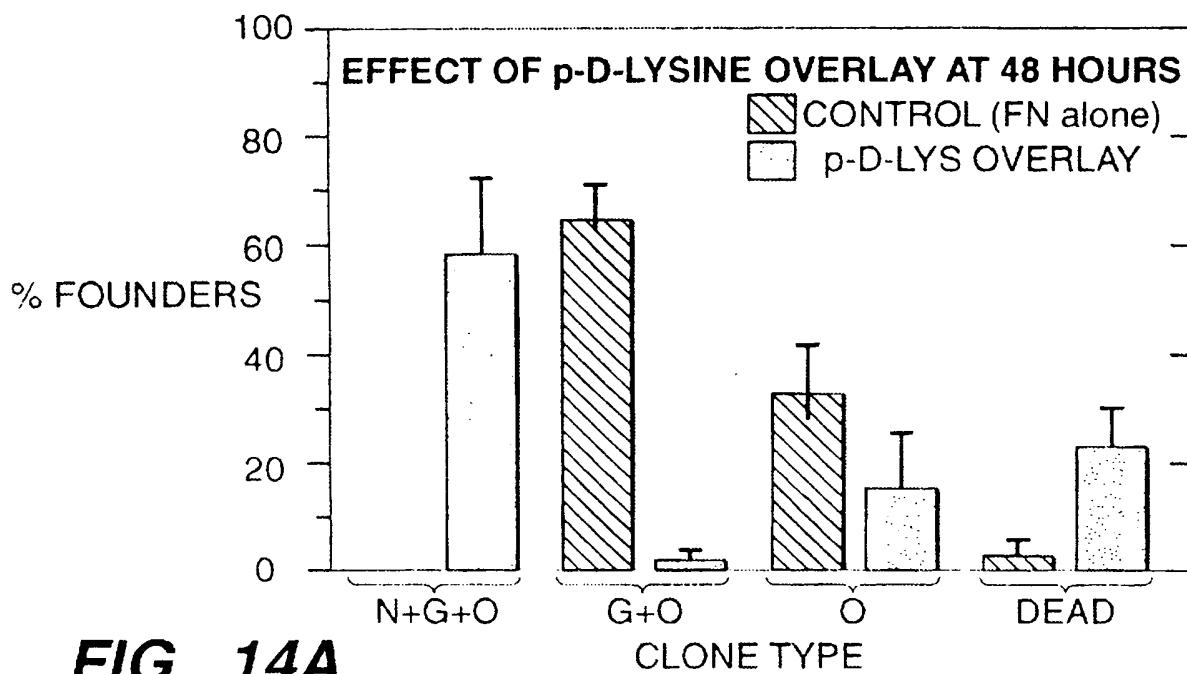


FIG._ 14A

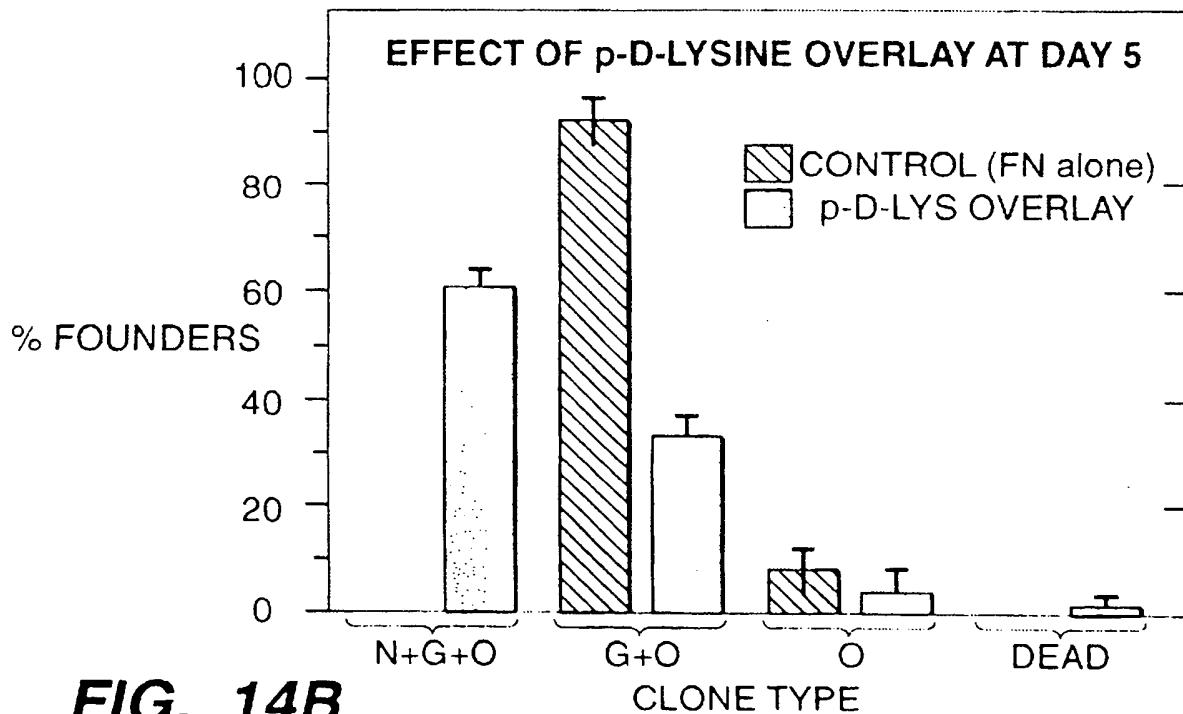


FIG._ 14B

SUBSTITUTE SHEET (RULE 26)

A

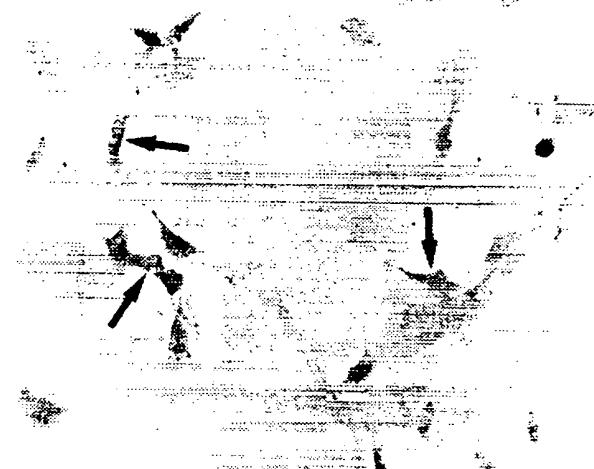


FIG._15A

B



FIG._15B

SUBSTITUTE SHEET (RULE 26)

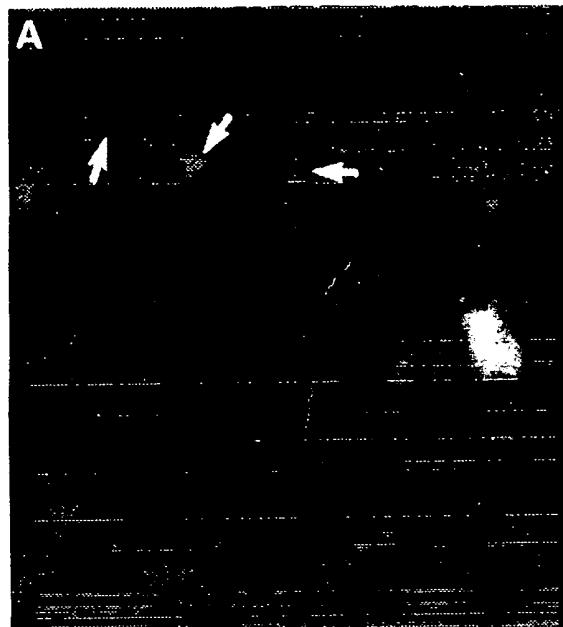


FIG._16A

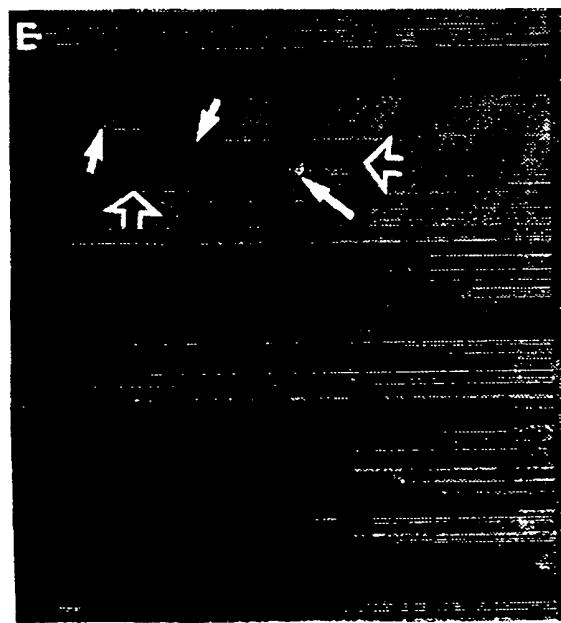


FIG._16B

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/07000

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 5	C12N5/06	C12N5/08	C12N5/10	C12P21/08	G01N33/569
G01N33/577					

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CELL vol. 71 , 11 December 1992 , CAMBRIDGE, NA US pages 973 - 985 DEREK L. STEMPLE ET AL. 'ISOLATION OF A STEM CELL FOR NEURONS AND GLIA FROM THE MAMMALIAN NEURAL CREST' see the whole document ---	1-43
X	WO,A,89 03872 (AMRAD CORPORATION LIMITED) 5 May 1989 see page 1, line 1 - page 5, line 29 ---	44-55
A	US,A,4 705 751 (DEANE F. MOSHER) 10 November 1987 see column 1, line 67 - column 2, line 12 ---	7,11 -/-

 Further documents are listed in continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

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- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

Date of the actual completion of the international search

17 December 1993

Date of mailing of the international search report

17 December 1993

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patendaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax (- 31-70) 340-3016	Rempp, G

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

Intern'l Application No
PCT/US 93/07000

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 707 448 (EUGENE O. MAJOR) 17 November 1987 see column 1, line 49 - line 68 -----	44

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/07000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8903872	05-05-89	AU-B- 600393 EP-A- 0383804	09-08-90 29-08-90
US-A-4705751	10-11-87	NONE	
US-A-4707448	17-11-87	NONE	

